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Award Number:
W81XWH-08-1-0367

TITLE:
Regulatory Role of the NF-kB Pathway in Lymphangiogenesis and Breast Cancer Metastasis

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CONTRACTING ORGANIZATION:
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REPORT DATE:
October 2010

TYPE OF REPORT:
Annual Summary

PREPARED FOR:
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

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1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-10-2010	Annual Summary	1 JUL 2008 - 30 SEP 2010

4. TITLE AND SUBTITLE
Regulatory Role of the NF-kB Pathway in Lymphangiogenesis and Breast Cancer Metastasis

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-08-1-0367

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Southern Illinois University School of Medicine

Springfield, IL, 62702

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Material Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14.ABSTRACT on next page.

15. SUBJECT TERMS
Breast cancer metastasis; Lymphatic; Lymphangiogenesis; Vascular endothelial growth factor receptor-3 (VEGFR-3); Prospero-related homeobox-1 (Prox1); Inflammation; Nuclear factor-kappaB (NF-κB)

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	53	**19b. TELEPHONE NUMBER** *(include area code)*

14. ABSTRACT

Elevation of VEGFR-3, the primary mediator of lymphangiogenesis (i.e., new lymphatic vessel formation), is frequently associated with inflammation related to chronic disease and cancer. In the latter case, VEGFR-3 dependent lymphangiogenesis induced by inflamed tumors increases the incidence of distant metastasis, leading to decreased patient survival. However, the molecular mechanisms underlying inflammation-induced VEGFR-3 elevation and lymphangiogenesis are currently unknown. Two potential candidate genes that may regulate expression of VEGFR-3 are Prox1, the primary mediator of embryonic lymphangiogenesis, and NF-κB, the key intracellular regulator of inflammation-induced transcription. We hypothesized that the key inflammatory mediator, NF-κB, regulates transcription of key mediators of lymphangiogenesis, VEGFR-3 and Prox1. We further hypothesized that inflammation-induced elevation of VEGFR-3 and Prox1 are essential steps required for robust lymphangiogenesis in response to inflammation. The three primary goals of this study were to (1) delineate the time-course of events leading to inflammation-induced lymphangiogenesis *in vivo*; (2) clone and characterize the VEGFR-3 promoter and identify factors regulating VEGFR-3 expression *in vitro*; and (3) characterize the lymphatic phenotype of NF-κB p50 knockout mice.

To begin testing these hypotheses, we used a mouse model of peritonitis to characterize induction of lymphangiogenesis and expression kinetics of NF-κB, Prox1 and VEGFR-3. *In vivo* time-course analysis of inflammation-induced lymphangiogenesis showed activation of NF-κB followed by sequential upregulation of Prox1 and VEGFR-3 that preceded lymphangiogenesis by 4 and 2 days, respectively. Characterization of the VEGFR-3 promoter by luciferase-reporter and ChIP assays showed direct activation by Prox1, NF-κB p50 and p65 transcription factors. This also revealed that Prox1 and NF-κB p50 bind in close proximity and synergistically activate the VEGFR-3 promoter. Characterization of p50 knockout mice revealed significantly decreased lymphatic vessel density in several organs that corresponded to reduced VEGFR-3 and Prox1 expression. Activation of NF-κB by inflammatory stimuli also elevated expression of NF-κB, Prox1 and VEGFR-3 in cultured lymphatic endothelial cells, which enhanced proliferation and migration in response to the VEGFR-3-specific ligand, VEGF-C152S. Collectively, our findings suggest that induction of the NF-κB pathway by inflammatory stimuli activates Prox1, and both NF-κB and Prox1 activate the VEGFR-3 promoter leading to increased receptor expression in lymphatic endothelial cells. This, in turn, enhances the responsiveness of pre-existing lymphatic endothelium to VEGFR-3 binding factors, VEGF-C and VEGF-D, ultimately resulting in robust lymphangiogenesis.

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Introduction:

Chronic inflammation is frequently associated with breast cancer development[1;2], progression, and metastasis, which is the leading cause of mortality in these patients[3]. Frequently, the formation of new lymphatic vessels, i.e., lymphangiogenesis, facilitates initial metastasis to regional lymph nodes. Strong correlative evidence links chronic inflammation to both increased lymphangiogenesis[4] and breast cancer metastasis5, but the direct mechanism(s) are largely unknown. The key protein that regulates lymphangiogenesis is vascular endothelial growth factor receptor-3 (VEGFR-3)[6], a tyrosine kinase receptor expressed primarily in lymphatic endothelial cells (LECs)[7]. VEGFR-3 signaling is activated upon binding of vascular endothelial growth factor (VEGF)-C or the related factor, VEGF-D[6]. In adulthood, lymphangiogenesis and elevated VEGFR-3 expression coincide with pro-inflammatory conditions including cancer[8], wound healing[9], and chronic inflammatory diseases. Increased lymphatic vessel density has been documented in chronic airway infection[10], psoriasis[11], arthritis[12] and corneal injury[13]. VEGF-C and VEGF-D are elevated during inflammation, being produced by a variety of cells residing at inflamed sites, including macrophages[10;14;15], dendritic cells, neutrophils[10], mast cells, fibroblasts[16] and tumor cells[15]. Inflammationinduced lymphatic hyperplasia and lymphangiogenesis are likely the result of increased VEGFR-3 expression that amplifies response to VEGF-C/-D. This is supported by observations that blocking VEGFR-3 signaling inhibits lymphangiogenesis during chronic inflammation[10], wound healing[17], and malignancy[18].

Lymphangiogenesis is also regulated by the lymphatic-specific transcription factor, Prospero-related homeobox-1 (Prox1)[19;20] that specifies lymphatic endothelial cell-fate by regulating VEGFR-3[20] and other lymphatic-specific proteins during embryogenesis. The central role of Prox1 in developmental lymphangiogenesis suggests a similar role for Prox1 in adulthood. Studies have shown that Prox1 induces VEGFR-3 expression in adult blood vascular endothelial cells (BECs)[19;21], whereas silencing Prox1 in adult LECs downregulates VEGFR-3

expression[21;22]. Prox1 has been shown to be upregulated by inflammatory cytokines[23] and to colocalize with VEGFR-3 in lymphatic vessels. However, the role of Prox1 in regulation of VEGFR-3 expression during inflammation *in vivo* is unknown. The primary mediators of the intracellular response to inflammation are dimeric transcription factors that belong to the nuclear factor-kappaB (NF-κB) family consisting of RelA (p65), NF-κB1 (p50), RelB, c-Rel, and NF-κB2 (p52)[24]. The main NF-κB protein complexes that regulate the transcription of responsive genes are p50/p65 heterodimers or p50 and p65 homodimers[25]. Over 450 NF-κB inducible genes have been identified, including proteins that mediate inflammation, immunity, tumorigenesis, and angiogenesis[24]. Several NF-κB-regulated genes stimulate lymphangiogenesis either directly (e.g., VEGF-A[26] and VEGF-C[27]) or indirectly, by upregulating VEGF-C and VEGF-D (e.g., IL-1β[16], TNF-α[16] and COX-2[15]). Activated NF-κB signaling coincides with increased VEGFR-3+ lymphatics during inflammation[10] suggesting a role for NF-κB in regulation of VEGFR-3 expression. In the work supported by this proposal we have identified one of the central molecular mechanisms underlying inflammation- and tumor-induced lymphangiogenesis. Our current data, based on *in vivo* and *in vitro* models of inflammation, suggest that Prox1 and VEGFR-3 are directly regulated by inflammation through NF-κB signaling. These important and novel findings have set the basis for future studies that will investigate the primary functions of these factors in promoting breast cancer lymphatic metastasis.

Body

Task 1. To determine the synergistic effects of inflammatory mediators (TNF-α, IL-1β, and IL-6) on proliferation, migration and survival of lymphatic endothelial cells (LEC) induced by VEGFR-3 ligands in vitro. To accomplish this task, we will:

A. Measure effect of lymphangiogenic factors VEGF-C (50ng/ml) or VEGF-D (50ng/ml) on induction of LEC proliferation in the presence or absence of TNF-α (10ng/ml), IL-1β (10ng/ml), or IL-6 (10ng/ml).
B. Measure effect of lymphangiogenic factors VEGF-C (50ng/ml) or VEGF-D (50ng/ml) on LEC migration in the presence or absence of TNF-α (10ng/ml), IL-1β (10ng/ml), or IL-6 (10ng/ml).
C. Measure effect of lymphangiogenic factors VEGF-C (50ng/ml) or VEGF-D (50ng/ml) on LEC survival in the presence or absence of TNF-α (10ng/ml), IL-1β (10ng/ml), or IL-6 (10ng/ml).

Data toward accomplishment of Task 1:

In this report we have accomplished the following preliminary studies necessary for completion of the goals outlined in Task 1

- cloned and characterized the human VEGFR-3 promoter
- demonstrated that NF-κB subunits p50 and p65 directly activate the VEGFR-3 promoter
- showed that NF-κB dependent inflammatory stimuli, IL-3 and LPS, upregulate endogenous VEGFR-3 expression in cultured LECs
- demonstrated that IL-3 and LPS induce LEC proliferation and migration
- showed that pretreatment with IL-3 and LPS enhances LEC proliferation and migration that is induced by the VEGFR-3 specific ligand, VEGF-C152S
- determined that NF-κB signaling is necessary for endogenous VEGFR-3 gene expression in LECs using sequence-specific and pharmacological NF-κB inhibitors

- demonstrated that primary mediators of lymphangiogenesis, Prox1 and VEGFR-3, are elevated during inflammatory lymphangiogenesis *in vivo*
- characterized the expression of inflammatory and lymphangiogenic mediators using a timecourse mouse model of inflammation
- investigated whether VEGFR-3 upregulation is necessary for inflammation-induced angiogenesis.

Collectively, we have found that inflammation-induced NF-κB signaling directly activates the VEGFR-3 promoter. We hypothesize that this, in turn, increases VEGFR-3 receptor density that leads to greater activation by VEGFR-3 ligands, VEGF-C and VEGF-D, resulting in a more robust lymphangiogenic response. Results and methods relating to task completion of Task 1 is provided in the peer reviewed journal article entitled "Inflammation induces lymphangiogenesis through up-regulation of VEGFR-3 mediated by NFkappaB and Prox1" (*Blood*. 2010 Jan 14;115(2):418-29) attached in appendix A of this report.

Task 2. To determine the effect of deficient NF-κB signaling on VEGFR-3 expression, lymphangiogenesis and tumor metastasis in an orthotopic breast cancer model in female p50 knockout mice. To accomplish this task, we will:

A. Implant syngeneic breast carcinoma cell lines (one non-metastatic and one highly metastatic derivative sub-line) in the MFP of wild-type (WT) and NF-κB p50 knockout mice (KO).
B. Assess the rate of tumor growth of breast cancer cell lines, tumor lymphangiogenesis, and lymphatic and distant metastasis.

Data toward accomplishment of Task 2

In this report we have accomplished the following preliminary studies necessary for completion of the goals outlined in Task 2A:

- Characterized lymphatic vessel density in p50 KO and WT mice showed that lymphatic vessel density in the liver, lungs, and MFP of KO mice was reduced by 40%, 14%, and 19%, respectively.
- Characterized expression of the primary pro-lymphangiogenic mediators, Prox1 and VEGFR-3, in p50 KO mice compared with WT.
- Showed 25-44% decreased VEGFR-3 and Prox1 expression in lymphatic endothelial cells of the lung and liver of p50 KO mice compared with WT, respectively.
- Demonstrated 25-50% decrease in Prox1 expression in non-lymphatic endothelial cells in the liver and brain.
- Despite reduced LVD in the MFP of p50 KO mice, there was a paradoxical increase in VEGFR-3 and Prox1 expression by 80-140% compared with in WT.
- Analysis of p65 and p52 NF-κB subunits and an array of inflammatory mediators showed a significant increase in p50 alternative pathways in the mammary fat pad (MFP) but not other organs, suggesting that compensatory mechanisms may regulate VEGFR-3 and Prox1 in the absence of NF-κB p50 in the MFP. However, the significantly reduced LVD in the MFP suggests that additional p50-dependent signals may be required for normal lymphangiogenesis.

During this analysis, we have made several novel observations such as: (i) original evidence for the role of NF-κB p50 in organ-specific maintenance of LVD and expression of the key pro-lymphangiogenic proteins, VEGFR-3 and Prox1, under normal conditions; (ii) strong associations among VEGFR-3, Prox1 and LVD that are consistent with the notion that NF-κB regulates Prox1 and VEGFR-3 expression in vivo; and (iii) novel evidence demonstrating the

suppression of Prox1 expression in p50 KO hepatocytes and neurons, suggesting for the first time that NF-κB p50 regulates Prox1 expression in non-lymphatic cell types. Although these data support the idea that NF-κB p50 is involved in regulation of VEGFR-3 and Prox1 expression, transcription of these genes might be additionally controlled by p65 and p52 as well as by other regulatory factors outside of the NF-κB family.

The lymphatics in the MFP of p50 KO mice were uniquely affected by the absence of NF-κB p50: similarly to lungs and liver, LYVE-1+ vessel density was significantly reduced, however, the expression of VEGFR-3 and Prox1 was paradoxically elevated. We drew two conclusions from these observations: (i) up-regulation of Prox1 and VEGFR-3 suggests compensatory overexpression of p50-alternative NF-κB factors; and (ii) normal MFP lymphangiogenesis might require p50-specific signals other than Prox1 and VEGFR-3. Results and methods relating to task completion of Task 2A is provided in the peer reviewed journal article entitled "Characterization of Prox1 and VEGFR-3 Expression and Lymphatic Phenotype in Normal Organs of Mice Lacking p50 Subunit of NF-κB" (*Microcirculation.* In press. Accepted 2010 Aug 19) and is attached in appendix B of this report.

Collectively, our data implicate a far more complex system regulating the expression of VEGFR-3 and induction of lymphangiogenesis in the MFP than was originally anticipated. Due to these setbacks, as well as, early graduation of the PI, we were unable to complete Task 2B.

Task 3. To determine the effect of anti-inflammatory treatment on VEGFR-3 expression, tumor lymphangiogenesis, lymphatic metastasis, and spread to distant organs in an orthotopic model of human breast carcinoma, MDA-MB-231. To accomplish this task, we will:

A. Implant luciferase tagged human breast carcinoma line MDA-MB-231 into the MFP of CB-17 SCID mice and treat them with NF-κB targeting anti-inflammatory drugs, PDTC and dexamethasone.

B. Assess tumor growth, tumor lymphangiogenesis, and lymphatic metastasis to distant organs.

Data toward accomplishment of Task 3

Due to graduation of the PI, we were unable to complete Task 3.

Key research accomplishments

Final Report

- Cloned and characterized nine human VEGFR-3 promoter-reporter constructs ranging from -849bp to -46bp relative to the transcription start site
- Identified NF-κB subunits p50 and p65 as key regulators of the VEGFR-3 promoter *in vitro* by using ChIP and promoter-reporter analyses
- Identified the lymphatic-specific transcription factor, Prox1, as a direct activator of the VEGFR-3 promoter *in vitro* by using ChIP and promoter-reporter analyses
- Discovered using a time-course model of inflammatory lymphangiogenesis that expression of Prox1 is rapidly increased by 1-2 days after the onset of inflammation and this is followed a 3-fold in VEGFR-3 protein expression 2-3 days later
- Found that upregulation of both Prox1 and VEGFR-3 precedes inflammation-induced formation of new lymphatic vessels
- Determined that the NF-κB dependent inflammatory stimuli, IL-3 and LPS, increase VEGFR-3 transcript expression by 4- to 6-fold
- Showed that increased VEGFR-3 expression in response to inflammatory mediators increases LEC responsiveness of to VEGFR-3 specific ligands.
- Characterized lymphatic vessel density in p50 KO and WT mice howed that lymphatic vessel density in the liver, lungs, and MFP of KO mice was reduced by 40%, 14%, and 19%, respectively.
- Characterized expression of the primary pro-lymphangiogenic mediators, Prox1 and VEGFR-3, in p50 KO mice compared with WT.
    - Showed 25-44% decreased VEGFR-3 and Prox1 expression in lung and liver of p50 KO mice compared with WT, respectively.
    - Despite reduced LVD in the MFP of p50 KO mice, there was a paradoxical increase in VEGFR-3 and Prox1 expression by 80-140% compared with in WT.

- Analysis of p65 and p52 NF-κB subunits and an array of inflammatory mediators showed a significant increase in p50 alternative pathways in the MFP but not other organs, suggesting that compensatory mechanisms may regulate VEGFR-3 and Prox1 in the absence of NF-κB p50 in the MFP. However, the significantly reduced LVD in the MFP suggests that additional p50-dependent signals may be required for normal lymphangiogenesis.

Reportable outcomes

Final Report

Degree obtained:

Ph.D. (Molecular Biology, Microbiology, and Biochemistry), August 2010, Department of Medical Microbiology, Immunology, and Cell Biology, Southern Illinois University School of Medicine, Springfield, Illinois

Peer Reviewed Journal Articles

1. **Michael J. Flister**, Lisa D. Volk, and Sophia Ran. Characterization of Prox1 and VEGFR-3 Expression and Lymphatic phenotype in Normal Organs of Mice Lacking p50 Subunit of NF-κB. *Microcirculation.* In press. Accepted 2010 Aug 19.
2. **Michael J. Flister**, Andrew Wilber, Kelly L. Hall, Caname Iwata, Kohei Miyazono, Riccardo E. Nisato, Michael S. Pepper, David C. Zawieja and Sophia Ran. Inflammation induces lymphangiogenesis through upregulation of VEGFR-3 mediated by NF-κB and Prox1. *Blood*. 2010 Jan 14;115(2):418-29.
3. Sophia Ran, Lisa Volk, Kelly Hall, and **Michael J. Flister**. Lymphangiogenesis and Lymphatic Metastasis in Breast Cancer. *Pathophysiology*. 2010 Sep;17(4):229-251.
4. Lisa D. Volk, **Michael J. Flister**, Christopher M. Bivens, Alan Stutzman, Neil Desai, Vuong Trieu and Sophia Ran (June 2008). Nab-paclitaxel efficacy in the orthotopic model of human breast cancer is significantly enhanced by concurrent anti-VEGF-A therapy. *Neoplasia.* 2008 Jun;10(6):613-23.

Awards/Presentations:

1. 2nd Place Presentation in 20th Annual Trainee Symposium, SIU School of Medicine, May 2010.
2. Selected to the U.S. Delegation for the 60th Meeting of Nobel Laureates in Lindau, Germany, June, 2010.

3. Bradley University Alumni Spotlight, March 2010
4. Best Student Publication Award for 2009.
5. 2nd Place Poster at Annual SIU SimmonsCooper Cancer Institute Symposium, SIU School of Medicine, September 2009.
6. 2nd Place Presentation in 19th Annual Trainee Symposium, SIU School of Medicine, May 2009.
7. Finalist AACR Scholar-in-Training Award for 2009 Annual Meeting, April 2009.
8. Selection for oral presentation, "VEGFR-3 and Prox1 are elevated by NF-κB during inflammatory lymphangiogenesis" Tumor biology: Mechanistic Approaches to Targeting Angiogenesis Minisymposium, American Association for Cancer Research Annual Meeting, Denver, CO, April 2009.
9. 1st Place Presentation in 18th Annual Trainee Research Symposium, SIU School of Medicine, April 2008.
10. Travel Award for American Association for Cancer Research Annual Meeting, San Diego, CA, April 2008. Awarded by SimmonsCooper Cancer Institute & Department of Medical Microgiology, Immunology, and Cell Biology, Southern Illinois University School of Medicine
11. Selection for oral presentation, **"**Prox1 and NF-κB directly regulate vascular endothelial growth factor receptor (VEGFR)-3 expression". Endothelial Progenitors: Induction of Angiogenesis Minisymposium, American Association for Cancer Research Annual Meeting, San Diego, CA, April 2008.

Published Abstracts:

1. **Flister MJ,** Hall K, Wilber A, and Ran S. Inflammatory mediators activate lymphatic endothelial cells (LECs) through elevation of NF-κB, Prox1, and VEGFR-3. American Association for Cancer Research Annual Meeting, Washington DC, 2010.
2. Hall K, **Flister MJ**, Volk L, Curry S, Wilber A, and Ran S. Angiopoietin-2 role in breast cancer metastasis. American Association for Cancer Research Annual Meeting, Washington DC, 2010.

3. **Flister MJ** and Ran S. VEGFR-3 and Prox1 are elevated by NF-κB during inflammatory lymphangiogenesis. 2nd Place Presentation in 19th Annual Trainee Symposium, SIU School of Medicine, May 2009.
4. **Flister MJ**, Wilber A, Hall KL, Iwata C, Miyazono K, Nisato RE, Pepper MS, Zawieja DC, and Ran S. VEGFR-3 and Prox1 are elevated by NF-κB during inflammatory lymphangiogenesis. American Association for Cancer Research Annual Meeting, Denver, CO, April 2009.
5. Volk L, Stutzman A, **Flister MJ**, Hall KL, Chihade D, Desai N, Trieu V, and Ran S. Mechanisms of nab-paclitaxel and bevacizumab cooperation in inhibition of breast tumor growth and metastasis. San Antonio Breast Cancer Symposium 2009.
6. **Flister MJ** and Ran S. Vascular endothelial growth factor receptor (VEGFR)-3 promoter activation by the inflammatory mediators NF-κB and Prox1. 18th Annual Trainee Research Symposium, SIU School of Medicine, Springfield, IL, April 2008.
7. **Flister MJ**, Zawieja DC, and Ran, S. NF-κB, Prox1 and inflammatory mediators regulate VEGFR-3 expression and lymphangiogenesis. Molecular Mechanisms in Lymphatic Function and Disease. Gordon Conference, Ventura, California, March 2008.
8. **Flister MJ**, Hall KL, Miele L, and Ran S. Prox1 and NF-κB directly regulate vascular endothelial growth factor receptor (VEGFR)-3 expression. American Association for Cancer Research Annual Meeting, San Diego, CA, April 2008.

Conclusions:

Conclusions of work towards completion of Task 1:

Here we have presented the first molecular evidence that VEGFR-3, the central mediator of lymphangiogenesis, is directly regulated by NF-κB transcription factors, p50 and p65, in response to extracellular inflammatory stimuli. We also present novel evidence that the lymphatic-specific transcription factor Prox1 is induced by NF-κB-dependent inflammation and elevation of both NF-κB and Prox1 preceded upregulation of VEGFR-3 by 2-3 days *in vivo*. *In vitro* we demonstrated that NF-κB and Prox1 transcription factors directly transactivate the VEGFR-3 promoter. Moreover, our data show that NF-κB dependent mediators, IL-3 and LPS, increased Prox1 and VEGFR-3 expression and responsiveness of LECs to the VEGFR-3 specific ligand, VEGF-C152S. Collectively, these results suggest that LEC stimulation by NF-κB dependent cytokines amplifies the lymphangiogenic signals by increasing VEGFR-3 expression.

Conclusions of work towards completion of Task 2:

This preliminary systematic comparison of VEGFR-3 and Prox1 expression in the lymphatics of normal organs in p50 KO and WT mice was important to lay the foundation for future experiments characterizing the effects of p50 deletion on breast cancer growth, lymphangiogenesis and lymphatic metastasis. During this analysis, we have made several novel observations that give broader implications to the functions of NF-κB, Prox1, and VEGFR-3 in normal vascular and non-vascular physiology. These include: 1) original evidence for the essential role of NF-κB p50 in organ-specific lymphatic development and expression of the key pro-lymphangiogenic proteins, VEGFR-3 and Prox1, under normal conditions; 2) strong associations among VEGFR-3, Prox1 and lymphatic vessel density that support the notion that NF-κB controls normal lymphatic vessel growth *in vivo* through regulation of Prox1 and VEGFR-3 expression; and 3) novel evidence demonstrating the suppression of Prox1 expression in p50 KO hepatocytes and neurons, suggesting for the first time that NF-κB p50 regulates Prox1

expression in cells other than LEC. Finally, these data advanced our basic understanding of molecular mechanisms governing postnatal lymphatic development and highlight the diverse roles of the NF-κB p50 subunit in normal physiology of both endothelial and non-endothelial tissues.

Reference List

1. Ben Baruch A. Host microenvironment in breast cancer development: inflammatory cells, cytokines and chemokines in breast cancer progression: reciprocal tumor-microenvironment interactions. *Breast Cancer Res.* 2003;5(1):31-36.
2. Yu JL, Rak JW. Host microenvironment in breast cancer development: inflammatory and immune cells in tumour angiogenesis and arteriogenesis. *Breast Cancer Res.* 2003;5(2):83-88.
3. Schoppmann SF, Horvat R, Birner P. Lymphatic vessels and lymphangiogenesis in female cancer: mechanisms, clinical impact and possible implications for anti-lymphangiogenic therapies (Review). *Oncol Rep.* 2002;9(3):455-460.
4. Mouta C, Heroult M. Inflammatory triggers of lymphangiogenesis. *Lymphat Res Biol.* 2003;1(3):201-218.
5. Chakrabarti R, Subramaniam V, Abdalla S, Jothy S, Prud'homme GJ. Tranilast inhibits the growth and metastasis of mammary carcinoma. *Anticancer Drugs.* 2009;20(5):334-345.
6. Veikkola T, Jussila L, Makinen T et al. Signalling via vascular endothelial growth factor receptor-3 is sufficient for lymphangiogenesis in transgenic mice. *EMBO J.* 2001;20(6):1223-1231.
7. Kaipainen A, Korhonen J, Mustonen T et al. Expression of the fms-like tyrosine kinase 4 gene becomes restricted to lymphatic endothelium during development. *Proc Natl Acad Sci U S A.* 1995;92(8):3566-3570.
8. Whitehurst B, Flister MJ, Bagaitkar J et al. Anti-VEGF-A therapy reduces lymphatic vessel density and expression of VEGFR-3 in an orthotopic breast tumor model. *Int J Cancer.* 2007;121(10):2181-2191.

9. Paavonen K, Puolakkainen P, Jussila L, Jahkola T, Alitalo K. Vascular endothelial growth factor receptor-3 in lymphangiogenesis in wound healing. *Am J Pathol.* 2000;156(5):1499-1504.
10. Baluk P, Tammela T, Ator E et al. Pathogenesis of persistent lymphatic vessel hyperplasia in chronic airway inflammation. *J Clin Invest.* 2005;115(2):247-257.
11. Kunstfeld R, Hirakawa S, Hong YK et al. Induction of cutaneous delayed-type hypersensitivity reactions in VEGF-A transgenic mice results in chronic skin inflammation associated with persistent lymphatic hyperplasia. *Blood.* 2004;104(4):1048-1057.
12. Zhang Q, Lu Y, Proulx ST et al. Increased lymphangiogenesis in joints of mice with inflammatory arthritis. *Arthritis Res Ther.* 2007;9(6):R118.
13. Maruyama K, Ii M, Cursiefen C et al. Inflammation-induced lymphangiogenesis in the cornea arises from CD11b-positive macrophages. *J Clin Invest.* 2005;115(9):2363-2372.
14. Cursiefen C, Chen L, Borges LP et al. VEGF-A stimulates lymphangiogenesis and hemangiogenesis in inflammatory neovascularization via macrophage recruitment. *J Clin Invest.* 2004;113(7):1040-1050.
15. Iwata C, Kano MR, Komuro A et al. Inhibition of cyclooxygenase-2 suppresses lymph node metastasis via reduction of lymphangiogenesis. *Cancer Res.* 2007;67(21):10181-10189.
16. Ristimaki A, Narko K, Enholm B, Joukov V, Alitalo K. Proinflammatory cytokines regulate expression of the lymphatic endothelial mitogen vascular endothelial growth factor-C. *J Biol Chem.*1998;273(14):8413-8418.
17. Goldman J, Rutkowski JM, Shields JD et al. Cooperative and redundant roles of VEGFR-2 and VEGFR-3 signaling in adult lymphangiogenesis. *FASEB J.* 2007;21(4):1003-1012.

18. Roberts N, Kloos B, Cassella M et al. Inhibition of VEGFR-3 activation with the antagonistic antibody more potently suppresses lymph node and distant metastases than inactivation of VEGFR-2. *Cancer Res.* 2006;66(5):2650-2657.

19. Hong YK, Harvey N, Noh YH et al. Prox1 is a master control gene in the program specifying lymphatic endothelial cell fate. *Dev Dyn.* 2002;225(3):351-357.

20. Wigle JT, Harvey N, Detmar M et al. An essential role for Prox1 in the induction of the lymphatic endothelial cell phenotype. *EMBO J.* 2002;21(7):1505-1513.

21. Petrova TV, Makinen T, Makela TP et al. Lymphatic endothelial reprogramming of vascular endothelial cells by the Prox-1 homeobox transcription factor. *EMBO J.* 2002;21(17):4593-4599.

22. Mishima K, Watabe T, Saito A et al. Prox1 induces lymphatic endothelial differentiation via integrin alpha9 and other signaling cascades. *Mol Biol Cell.* 2007;18(4):1421-1429.

23. Groger M, Loewe R, Holnthoner W et al. IL-3 induces expression of lymphatic markers Prox-1 and podoplanin in human endothelial cells. *J Immunol.* 2004;173(12):7161-7169.

24. Karin M. Nuclear factor-kappaB in cancer development and progression. *Nature.* 2006;441(7092):431-436.

25. Beinke S, Ley SC. Functions of NF-kappaB1 and NF-kappaB2 in immune cell biology. *Biochem J.* 2004;382(Pt 2):393-409.

26. Kiriakidis S, Andreakos E, Monaco C et al. VEGF expression in human macrophages is NF-kappaBdependent: studies using adenoviruses expressing the endogenous NF-kappaB inhibitor IkappaBalpha and a kinase-defective form of the IkappaB kinase 2. *J Cell Sci.* 2003;116(Pt 4):665-674.

27. Tsai PW, Shiah SG, Lin MT, Wu CW, Kuo ML. Up-regulation of vascular endothelial growth factor C in breast cancer cells by heregulin-beta 1. A critical role of p38/nuclear factor-kappa B signaling pathway. *J Biol Chem.* 2003;278(8):5750-5759.

Appendix A

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blood

2010 115: 418-429
Prepublished online Nov 9, 2009;
doi:10.1182/blood-2008-12-196840

Inflammation induces lymphangiogenesis through up-regulation of VEGFR-3 mediated by NF-{kappa}B and Prox1

Michael J. Flister, Andrew Wilber, Kelly L. Hall, Caname Iwata, Kohei Miyazono, Riccardo E. Nisato, Michael S. Pepper, David C. Zawieja and Sophia Ran

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Blood (print ISSN 0006-4971, online ISSN 1528-0020), is published semimonthly by the American Society of Hematology, 1900 M St, NW, Suite 200, Washington DC 20036.
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Inflammation induces lymphangiogenesis through up-regulation of VEGFR-3 mediated by NF-κB and Prox1

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The concept of inflammation-induced lymphangiogenesis (ie, formation of new lymphatic vessels) has long been recognized, but the molecular mechanisms remained largely unknown. The 2 primary mediators of lymphangiogenesis are vascular endothelial growth factor receptor-3 (VEGFR-3) and Prox1. The key factors that regulate inflammation-induced transcription are members of the nuclear factor-kappaB (NF-κB) family; however, the role of NF-κB in regulation of lymphatic-specific genes has not been defined. Here, we identified VEGFR-3 and Prox1 as downstream targets of the NF-κB pathway. In vivo time-course analysis of inflammation-induced lymphangiogenesis showed activation of NF-κB followed by sequential up-regulation of Prox1 and VEGFR-3 that preceded lymphangiogenesis by 4 and 2 days, respectively. Activation of NF-κB by inflammatory stimuli also elevated Prox1 and VEGFR-3 expression in cultured lymphatic endothelial cells, resulting in increased proliferation and migration. We also show that Prox1 synergizes with the p50 of NF-κB to control VEGFR-3 expression. Collectively, our findings suggest that induction of the NF-κB pathway by inflammatory stimuli activates Prox1, and both NF-κB and Prox1 activate the VEGFR-3 promoter leading to increased receptor expression in lymphatic endothelial cells. This, in turn, enhances the responsiveness of pre-existing lymphatic endothelium to VEGFR-3 binding factors, VEGF-C and VEGF-D, ultimately resulting in robust lymphangiogenesis. (Blood. 2010;115:418-429)

Introduction

The lymphatic vascular system has multiple functions in normal physiology including regulation of interstitial pressure,[1] lipid absorption,[2] immune surveillance,[1] and resolution of inflammation.[3] The formation of new lymphatic vessels (ie, lymphangiogenesis) is dynamic during embryogenesis but is relatively rare and selectively regulated in adulthood. Insufficient development of postnatal lymphatic vessels impairs immune function and causes tissue edema,[1] whereas excessive lymphangiogenesis is associated with malignancy and strongly implicated in lymphatic metastasis.[4]

The key protein that regulates lymphangiogenesis is vascular endothelial growth factor receptor-3 (VEGFR-3),[5] a tyrosine kinase receptor expressed primarily in lymphatic endothelial cells (LECs).[6] VEGFR-3 signaling is activated upon binding of vascular endothelial growth factor-C (VEGF-C) or the related factor, VEGF-D.[5] In adulthood, lymphangiogenesis and elevated VEGFR-3 expression coincide with inflammatory conditions including cancer,[7] wound healing,[8] and chronic inflammatory diseases. Increased lymphatic vessel density (LVD) has been documented in chronic airway infection,[9] psoriasis,[10] arthritis,[11] and corneal injury.[12] VEGF-C and VEGF-D are elevated during inflammation, being produced by a variety of cells residing at inflamed sites, including macrophages,[9,13,14] dendritic cells, neutrophils,[9] mast cells, fibroblasts,[15] and tumor cells.[14] Inflammation-induced lymphatic hyperplasia and lymphangiogenesis are likely the result of increased VEGFR-3 expression that amplifies response to VEGF-C/-D. This is supported by observations that blocking VEGFR-3 signaling inhibits lymphangiogenesis during chronic inflammation,[9] wound healing,[16] and malignancy.[17]

Lymphangiogenesis is also regulated by the lymphatic-specific transcription factor, Prospero-related homeobox-1 (Prox1)[18,19] that specifies LEC fate by regulating expression of VEGFR-3[19] and other lymphatic-specific proteins during embryogenesis. The central role of Prox1 in developmental lymphangiogenesis suggests a similar role for Prox1 in adulthood. Studies have shown that Prox1 induces VEGFR-3 in adult blood vascular endothelial cells (BECs),[18,20] whereas silencing Prox1 in adult LECs down-regulates VEGFR-3 expression.[20,21] Prox1 has been shown to be up-regulated by inflammatory cytokines[22] and to colocalize with VEGFR-3 in lymphatic vessels. However, the role of Prox1 in regulation of VEGFR-3 expression during inflammation in vivo is unknown.

The primary mediators of the inflammatory response are dimeric transcription factors that belong to the nuclear factor-kappaB (NF-κB) family consisting of RelA (p65), NF-κB1 (p50), RelB, c-Rel, and NF-κB2 (p52).[23] The main NF-κB protein complexes that regulate the transcription of responsive genes are p50/p65 heterodimers or p50 and p65 homodimers.[24] More than

Submitted December 29, 2008; accepted October 13, 2009. Prepublished online as *Blood* First Edition paper, November 9, 2009; DOI 10.1182/blood-2008-12-196840.

The online version of this article contains a data supplement.

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450 NF-κB–inducible genes have been identified, including proteins that mediate inflammation, immunity, tumorigenesis, and angiogenesis.[23] Several NF-κB–regulated genes stimulate lymphangiogenesis either directly (eg, VEGF-A[25] and VEGF-C[26]) or indirectly, by up-regulating VEGF-C and VEGF-D (eg, IL-1β,[15] TNF-α,[15] and COX-2[14]). Activated NF-κB signaling coincides with increased VEGFR-3$^+$ lymphatics during inflammation,[9] suggesting a role for NF-κB in regulation of VEGFR-3 expression.

Although extensive evidence supports the link between inflammation and lymphangiogenesis, the molecular mechanisms underlying this association are largely unknown. We postulate that NF-κB, the main intracellular mediator of inflammation, regulates transcription of key mediators of lymphangiogenesis, VEGFR-3 and Prox1. To test this hypothesis, we used a mouse model of inflammatory peritonitis,[14] which showed that lymphangiogenesis is preceded by increased VEGFR-3 and Prox1 expression on preexisting inflamed lymphatic vessels. Analysis of the human VEGFR-3 promoter showed transcriptional regulation by p50, p65, and Prox1. These data demonstrate for the first time that NF-κB and Prox1 induce VEGFR-3 transcription, indicating the important roles for both factors in the regulation of VEGFR-3–dependent inflammatory lymphangiogenesis in vivo.

Methods

Materials

Human Prox1 CDS ligated into pCMV6-XL6 (pCMV-Prox1) plasmid was purchased from OriGene. NF-κB plasmids, pCMV-Flag-p50 and pCMV-Flag-p65, were kindly provided by Dr Albert Baldwin (University of North Carolina, Chapel Hill). Promoter-luciferase reporter plasmids for ubiquitin C and phosphoglycerate kinase were described previously.[27] Lipopolysaccharide (LPS) was purchased from Sigma-Aldrich. Rat VEGFR-3–specific ligand, VEGF-C152S, and human interleukin-3 (IL-3) were purchased from Peprotech. Pyrrolidine dithiocarbamate (PDTC) and MG-132 were purchased from Calbiochem. Leptomycin B was from LC Laboratories.

Antibodies

Primary antibodies used in this study were: goat anti–mVEGFR-3 and anti-Prox1 (R&D Systems); rabbit anti-p65, anti-pp65, anti-p50, and anti-pp50 (Santa Cruz); rabbit anti–mLYVE-1 and anti-Prox1 (AngioBio); rabbit anti–Ki-67 (Biomeda); goat anti–acetylated-histone-H3 (Upstate); mouse anti-Flag (ABM); mouse anti–β-actin (JLA20; Developmental Studies Hybridoma Bank); and rabbit anti–VEGF-C (Invitrogen). Secondary horseradish peroxidase–, fluorescein isothiocyanate–, and Cy3-conjugated donkey anti–rabbit and anti–goat antibodies and nonspecific rabbit immunoglobulin G (IgG) were from Jackson ImmunoResearch Laboratories.

Cell lines

Rat LECs (RLECs) were isolated and cultured as previously described.[28] Human embryonic kidney cells (HEK293) were cultured in Dulbecco modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS). Human primary LECs (H-LLY) and immortalized human dermal LECs ($HDLEC_{htert}$)[29] were cultured in gelatin-coated flasks in microvascular endothelial cell growth medium-2 (EGM-2MV) medium (Clonetics). Human lung microvascular endothelial cells (HULECs) were obtained from the Centers for Disease Control and Prevention.

Mouse peritonitis model

All mice experiments were approved by Southern Illinois University School of Medicine Institutional Laboratory Animal Care and Use Committee. Female BALB/c mice (3-6 months) were obtained from The Jackson Laboratory and treated in accordance with institutional guidelines. Peritonitis was induced by 0.5-mL intraperitoneal injections of 1.5% sodium thioglycollate (vol/vol in saline; BD Biosciences) for 2 weeks, as previously described.[14] For time-course analysis, mice (3-4 per group) received thioglycollate (TG) every 48 hours for the indicated periods. Control mice were injected intraperitoneally with 0.5 mL of saline. Diaphragms were removed after a 2-week treatment, fixed with 10 N of Mildform for 1 hour at room temperature, bathed in 30% sucrose overnight, and snap-frozen.

Immunohistochemistry

Frozen 8-μm sections were fixed with acetone for 10 minutes, washed in phosphate-buffered saline plus Tween-20 (pH 7.4, 0.1% Tween-20) for 10 minutes and incubated for 1 hour at 37°C with primary antibodies (diluted 1:100) against VEGFR-3, LYVE-1, Prox1, or Ki-67, followed by appropriate fluorescein isothiocyanate– or Cy3-conjugated secondary antibodies (diluted 1:100) for 1 hour at 37°C. For double immunofluorescent staining, sections were incubated with each primary and secondary antibody for 1 hour at 37°C and washed for 10 minutes in phosphate-buffered saline plus Tween-20 between steps. Slides were mounted in Vectashield medium containing 4,6′-diamidino-2-phenylindole (4,6 diamidino-2-phenylindole) nuclear stain (Vector Labs). Images were acquired on an Olympus BX41 upright microscope equipped with a DP70 digital camera and DP Controller software (Olympus).

Immunofluorescent intensity measurements

Analysis of VEGFR-3 and LYVE-1 double-staining was performed as described by Tammela et al,[30] with slight modifications. In brief, diaphragm sections were double-stained with goat anti–mVEGFR-3 and rabbit anti–mLYVE-1 antibodies. Fluorescent images were acquired at a constant exposure time at 400× magnification on an Olympus IX71 inverted microscope (Olympus) equipped with a Retiga Exi charge-coupled device camera (QImaging). Diaphragms stained with secondary antibodies alone were used to set the exposure time. Images acquired at a constant exposure time were converted to 12-bit gray scale followed by outlining vascular structures and analysis with Image-Pro Software (Media Cybernetics). Supplemental Figure 1 (available on the *Blood* website; see the Supplemental Materials link at the top of the online article) shows an example of image acquisition and vessel outlining. All images were within a linear intensity range between 0 and 4095. To exclude nonspecific staining, structures less than 10 μm (1 μm = 6.4 pixels) in diameter were excluded. To calculate mean vessel intensity, the sums of pixel intensities per vessel were divided by total vessel area (μm^2). Mean vessel intensities from 5 to 10 images per diaphragm (n = 3 per group) were averaged and compared between treated and control groups.

Western blot analysis

Cells were lysed in ice-cold buffer [50mM tris (hydroxymethyl)aminomethane-HCl, pH 7.5, 150mM NaCl, 1mM ethylenediaminetetraacetic acid, 1% Triton-X100, 0.1% sodium dodecyl sulfate, phenylmethylsulphonyl fluoride 1:100, and protease inhibitor cocktail 1:50]. Proteins separated in 12% sodium dodecyl sulfate–polyacrylamide gel were transferred onto nitrocellulose membranes followed by overnight incubation with primary antibodies against p50, p65, Prox1, LYVE-1, VEGFR-3, Flag-tag, or β-actin; 1-hour incubation with horseradish peroxidase–conjugated secondary antibodies; and development with enhanced chemiluminescence reagent (Pierce). Protein bands were visualized using a Fujifilm LAS-3000 camera and analyzed with Image-Reader LAS-3000 software.

VEGFR-3 promoter cloning

Segments of −849 bp, −514 bp, −341 bp, −331 bp, −118 bp, and −46 bp of the 5′ untranslated region of VEGFR-3 and +55 bp of exon 1 were amplified by polymerase chain reaction (PCR) from a human genomic bacterial artificial chromosome (BAC) clone, CTD-2546M13 (Open Biosystems). Promoter segments spanning −436/−254 bp and −849/−254 bp (Δ309) were also PCR-amplified. Products were cloned into the pGL4 basic vector (Promega) to produce VEGFR-3 promoter-luciferase constructs. All

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clones were sequenced and verified through comparison with published genomic sequence. Human VEGFR-3 promoter sequence (GenBank accession no. DQ911346[31]) was analyzed using MatInspector (http://www.genomatix.de/products/MatInspector/index.html[32]) and compared with published transcription factor binding sites.

Assay for VEGFR-3 promoter activity

Cells were transfected with 1 μg of DNA composed of 0.96 μg of promoter construct and 0.04 μg of Herpes simplex thymidine-kinase promoter–driven renilla luciferase (Promega) mixed with 3 μL of ExGen500 (Fermentas). After 24 hours, cells were lysed with 0.2% Triton-X100, and firefly and renilla luciferase activities were measured by a dual-luciferase assay performed according to manufacturer's protocol. Promoter-firefly luciferase activity was normalized per renilla activity or milligram of total protein.

Inflammatory stimulation of LECs

$HDLECs_{htert}$ were seeded in 6-well plates (200 000 cells/well) in 0.5% EGM2 medium (Lonza). Medium was replaced daily during a 72-hour time period, before treatment with IL-3 (10 ng/mL) or LPS (100 ng/mL) for 6 or 24 hours. RNA extraction and analysis of transcripts by quantitative reverse-transcription (qRT)–PCR was performed as described in "RT-PCR and qRT-PCR."

Cell proliferation and migration assays

RLECs were seeded in DMEM containing 1.5% FBS in 24-well plates at the density of 50 000 cells/well. IL-3 (5-100 ng/mL), LPS (50-1000 ng/mL), and VEGF-C152S (25-200 ng/mL) were added 2 hours after seeding. The effect of combined cytokines was measured after stimulation with VEGF-C152S (100 ng/mL) mixed with IL-3 (10 ng/mL) or LPS (500 ng/mL). After 72-hour incubation, cells were trypsinized and enumerated. The results are presented as the averaged cell number per well derived from 3 experiments performed in triplicate plus or minus SEM.

Cell migration was measured using 8 μm-pore Transwells according to the manufacturer's protocol (Corning). In brief, 50 000 RLECs were seeded in 0.25% DMEM on pre-equilibrated inserts. IL-3 (10 ng/mL), LPS (500 ng/mL), VEGF-C152S (200 ng/mL), or 0.25% FBS (negative control) was added to bottom chambers. After 24-hour incubation, inserts were washed, fixed in 2% paraformaldehyde for 10 minutes, and stained by crystal violet. Numbers of cells migrated per field were determined on 6 random images acquired at 200× and averaged.

ChIP

RLECs (2×10^7) were grown to 90% confluence and fixed with 1% formaldehyde. Cell lysis, shearing, and chromatin immunoprecipitation (ChIP) were performed using a ChIP-IT Express Kit according to the manufacturer's protocol (Active Motif). Chromatin was precipitated with anti-p50, p65, Prox1, acetylated-histone-H3 antibodies, or nonspecific rabbit IgG (negative control). Precipitated chromatin was amplified by PCR using primers for rat VEGFR-3 promoter listed in supplemental Table 1.

Suppression of p50/p65 expression by siRNA

Previously validated 21-nucleotide-long siRNA duplexes against p50 (sense strand, 5′-GGGGCUAUAAUCCUGGACUdTdT-3′)[33] and p65 (sense strand, 5′-GCCCUAUCCCUUUACGUCAdTdT-3′)[34] (Dharmacon) and predesigned Silencer Negative Control no. 1 siRNA (Ambion) were used for suppression of p50 and p65 expression. H-LLY cells were transfected with siRNA for 16 hours using siPORT NeoFX (Ambion) according to the manufacturer's protocol. Total RNA was isolated 48 hours after transfection and transcript levels were determined by qRT-PCR.

RT-PCR and qRT-PCR

Total RNA extracted by Tri-reagent was reverse transcribed using RTG You-Prime Reaction beads (Amersham) and random hexamer primers (Invitrogen). All primers used in this study are listed in supplemental Table 1. End point RT-PCR analysis was performed as previously described,[7] then visualized and analyzed using a FluroChem5500 imager (AlphaInnotech). qRT-PCR was performed using SYBR Master Mix and a 7500 Real-Time PCR machine from Applied Biosystems. Data were normalized to β-actin and relative mRNA expression was determined using the ΔΔCt method.

Statistical analysis

Statistical analysis was performed using SAS software (SAS Institute Inc). All results are expressed as mean plus or minus SEM. Differences in lymphatic vessel densities between groups were assessed by unpaired Student *t* test or Wilcoxon rank sum test. Intensity of VEGFR-3 and LYVE-1 staining per lymphatic vessels was assessed by analysis of variance for a nested design. Statistical significance was defined as *P* value less than .05.

Results

Inflammation induces lymphatic VEGFR-3 and Prox1 expression during lymphangiogenesis in vivo

Regulation of VEGFR-3 by inflammation is suggested by reports demonstrating inhibition of lymphangiogenesis by blockade of VEGFR-3 signaling.[9] Prox1 may also contribute to this process because it is induced by inflammatory mediators,[22] which coincides with elevated VEGFR-3.[35] However, the roles of VEGFR-3 and Prox1 in inflammatory lymphangiogenesis have not been demonstrated.

To analyze VEGFR-3 and Prox1 expression during inflammation, we induced peritonitis in Balb/c mice by TG injections, a method reported to induce lymphangiogenesis in the diaphragm.[14] Diaphragms from saline (control)– and TG-treated mice were removed after a 2-week treatment and stained for the lymphatic marker, LYVE-1, and VEGFR-3 (Figure 1). Consistent with previous studies,[14,36] the number of $LYVE\text{-}1^+$ lymphatic vessels increased by 1.9-fold (± 0.3-fold) in TG-treated mice compared with controls (Figure 1A-B). These tissues also showed a 4.5-fold (± 0.3-fold) increase in $VEGFR\text{-}3^+$ vessel density (Figure 1A,C). Coexpression of VEGFR-3 and LYVE-1 was quantified using the method described by Tammela et al[30] that measures the fluorescent intensity of target expression normalized per vascular area. This revealed a lymphatic-specific increase of VEGFR-3 (~ 67%) but not LYVE-1 (Figure 1D-E, supplemental Figure 3) expression, suggesting that inflammation increases both LVD and VEGFR-3 expression per individual vessel.

Prox1 reportedly regulates VEGFR-3 expression in cultured LECs[18,20]; however, a similar function in vivo has not been reported. We sought to determine Prox1 expression in $VEGFR\text{-}3^+$ lymphatic vessels during inflammation. Double-staining showed coincident up-regulation of VEGFR-3 and Prox1 in lymphatic vessels of inflamed diaphragms compared with control tissues (Figure 1F). Moreover, the frequency of $Prox1^+$ nuclei per lymphatic vessel area was increased by 3.3-fold (± 0.5-fold; Figure 1G).

To determine the proliferative status of $VEGFR\text{-}3^+/Prox1^+$ vessels, control and inflamed sections were costained for Ki-67 in combination with anti–VEGFR-3 or anti-Prox1 antibodies. Quiescent lymphatic vessels of control mice lacked Ki-67. In contrast, lymphatic vessels in TG-treated mice displayed widespread Ki-67 colocalized with both Prox1 and VEGFR-3 (Figure 1H). Collectively, these data demonstrate that inflammation induces VEGFR-3 and Prox1 expression on preexisting and sprouting lymphatic vessels.

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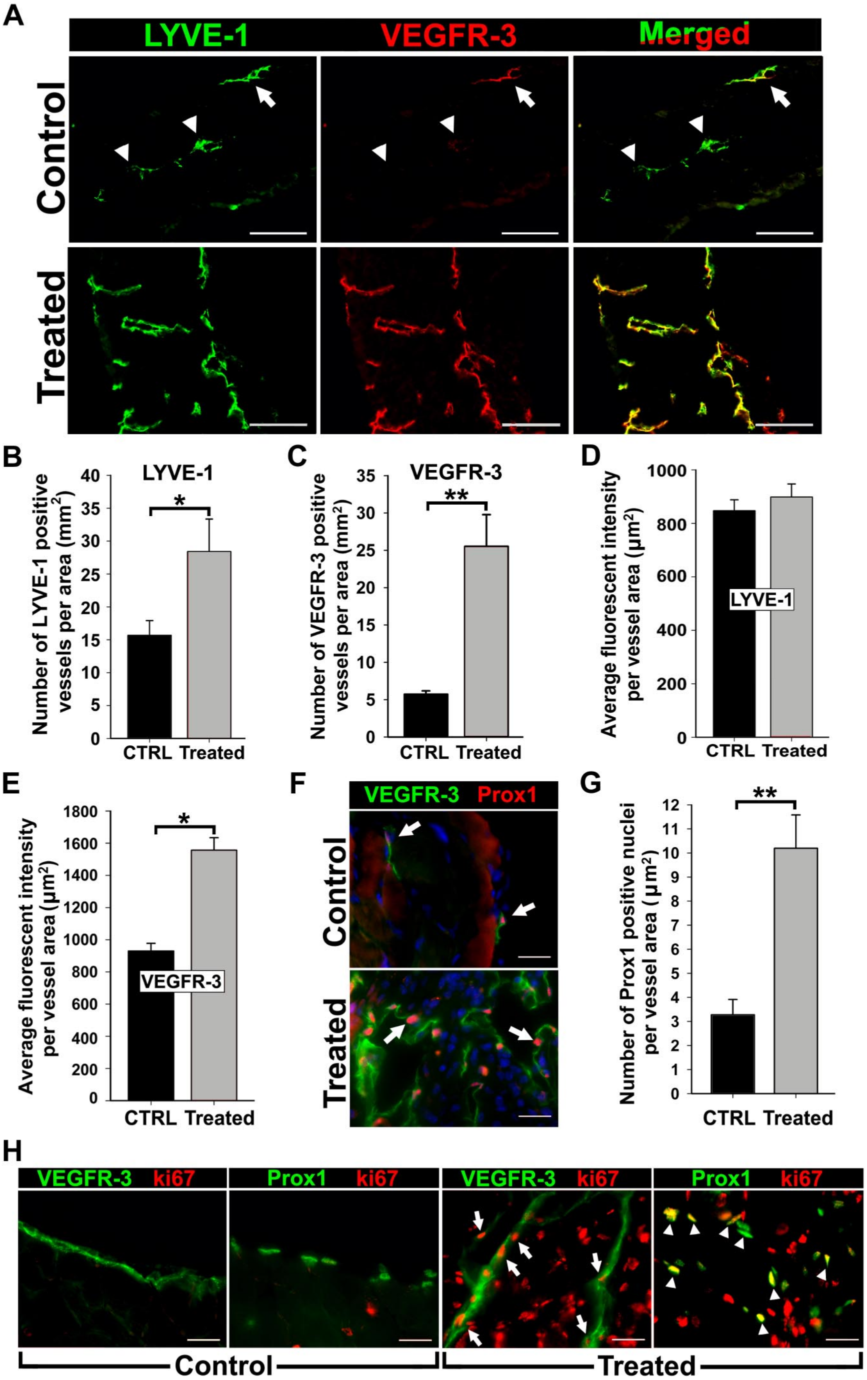

Figure 1. Inflammation induces VEGFR-3 and Prox1 expression in activated lymphatic vessels. Peritonitis was induced by repetitive intraperitoneal injections of thioglycollate (TG) every 48 hours for 2 weeks. (A) Diaphragms from mice treated for 2 weeks with TG to induce peritonitis or saline as a control (n = 3 mice per group) were double-stained with anti–LYVE-1 and anti–VEGFR-3 antibodies. Note strong expression and complete overlap of VEGFR-3 with LYVE-1 in inflamed tissues compared with quiescent lymphatic vessels in control sections with weakly detected (arrow) or absent (arrowheads) VEGFR-3. LYVE-1$^+$ (B) and VEGFR-3$^+$ (C) lymphatic vessels were counted on the entire diaphragm sections and the numbers were normalized per total section area expressed in square millimeters. The results are presented as the mean vessel density per group ± SEM. (B) *$P < .05$ versus control as determined by Wilcoxon rank sum test. (C) **$P < .01$ versus control as determined by Student unpaired t test. The mean fluorescent intensity (MFI) per vessel was analyzed on LYVE-1$^+$ (D) and VEGFR-3$^+$ (E) lymphatic vessels (5-10 vessels per diaphragm). MFI is expressed as relative units normalized per vascular area expressed in square micrometers. The mean MFI values ± SEM derived from 3 mice per group are shown. (E) *$P < .05$ versus control, as determined by nested analysis of variance described in "Statistical analysis." (F) Diaphragms from TG-treated and control mice were double-stained with anti-Prox1 and anti–VEGFR-3 antibodies. Arrows point to Prox1$^+$ nuclei. (G) Prox1$^+$ nuclei were enumerated and normalized per LYVE-1$^+$ lymphatic area (μm^2) in diaphragms of TG- and saline-treated control mice. **$P < .01$ versus control as determined by Student unpaired t test. (H) Diaphragm sections were costained with antibodies against VEGFR-3 or Prox1 and a proliferative marker, Ki-67, to assess proliferative status of lymphatic vessels in the diaphragms of TG-treated or control mice. Note overlapping expression of Ki-67/VEGFR-3 (arrow) and Ki-67/Prox1 (arrowhead) detected in inflamed lymphatic vessels but absent from quiescent lymphatic vessels in control tissues. Scale bars represent 100 μm (A) and 20 μm (F,H).

Increased Prox1 and VEGFR-3 expression precedes lymphangiogenesis

LEC activation is associated with increased Prox1[37] and VEGFR-3,[5] yet their lymphatic-specific expression kinetics at early stages of lymphangiogenesis has not been examined. To determine the timeline of events leading to lymphangiogenesis, diaphragms from control and TG-treated mice harvested at days 1 to 4 and 7 after treatment were analyzed for expression of Prox1, VEGFR-3, and LYVE-1 by immunofluorescence and Western blot. Figure 2A and B show that compared with control tissues, the density of Prox1$^+$ lymphatic vessels increased (3.8-fold, $P < .001$) on the first day and remained significantly elevated (2.2- to 3.1-fold) on days 2 to 7. In contrast, the increase in VEGFR-3$^+$ vessel density became statistically significant only on day 4 (1.7-fold, $P < .05$) and day 7 (3.1-fold, $P < .01$, Figure 2C). During this period, LYVE-1$^+$ vessel density was unchanged except for an insignificant 1.6-fold increase on day 7 (Figure 2D). This immunofluorescent analysis showed that increased Prox1 expression precedes VEGFR-3 up-regulation by 2 to 3 days and elevation of both proteins precedes lymphangiogenesis.

Western blot analysis of actin-normalized protein expression of lymphatic markers as well as total and phosphorylated NF-κB p50 and p65 at different days after treatment confirmed this conclusion (Figure 2E). As expected, p50, p65, p-p50, and p-p65 were induced by inflammation with the most pronounced changes detected in p-p50 on the first day of treatment. NF-κB increase was mirrored

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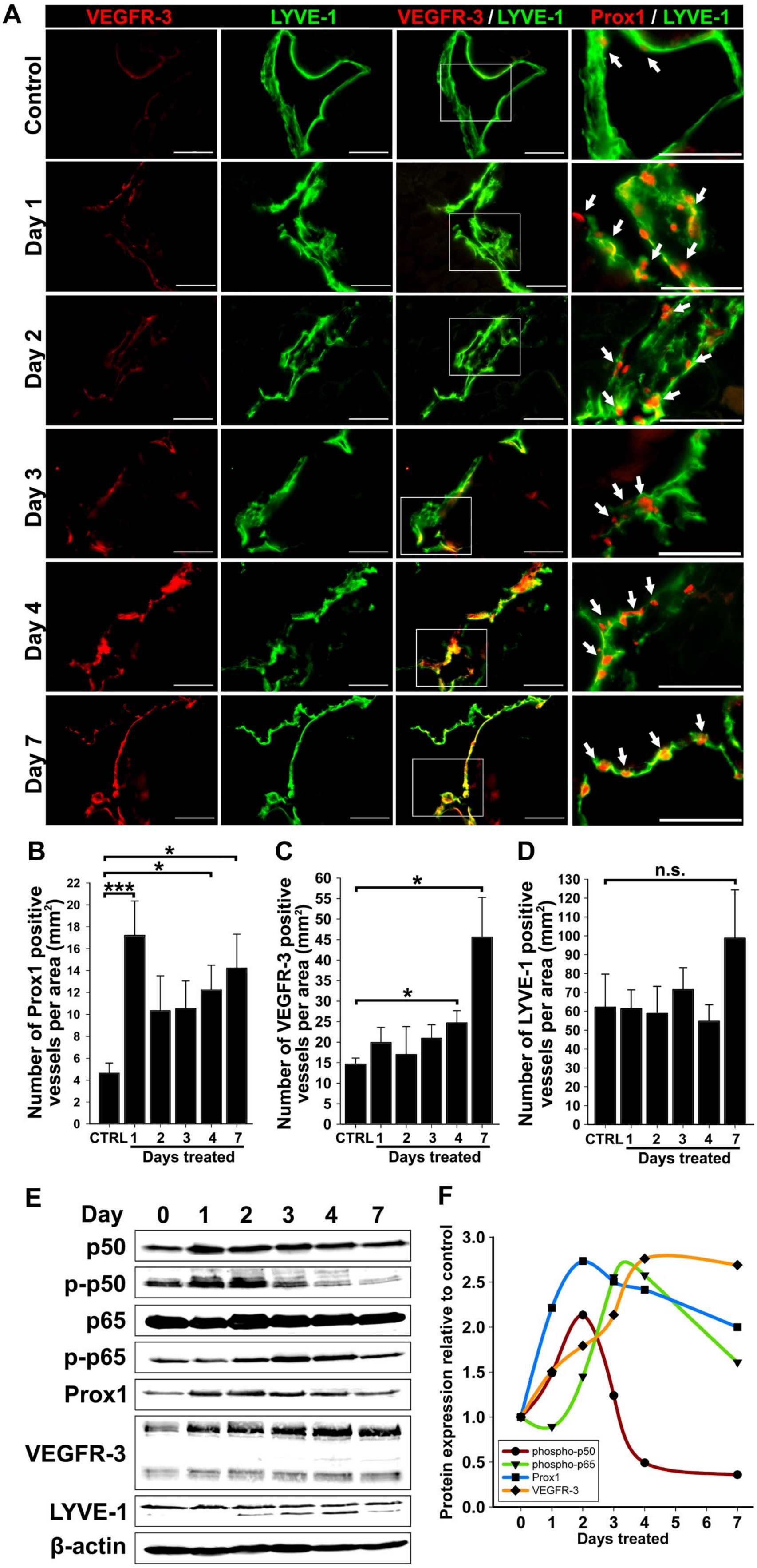

Figure 2. Up-regulation of VEGFR-3 and Prox1 precedes new lymphatic vessel formation during inflammation. (A) Double immunostaining of VEGFR-3/LYVE-1 and Prox1/LYVE-1 in serial diaphragm sections derived from mice treated with saline or TG (n = 3-4 mice per group) and harvested 1, 2, 3, 4, and 7 days after onset of treatment. Scale bars represent 50 μm. Lymphatic vessels shown are representative of whole diaphragm sections from 3 to 4 mice per group. (B-D) Quantification of Prox1-positive (B), VEGFR-3—positive (C), and LYVE-1—positive (D) vessels normalized per area of the entire diaphragm section measured in square millimeters. Quantitative analysis was performed on diaphragms harvested from 3 to 4 mice per group at indicated days after the first TG or saline injection. Data are presented as the mean number of vessels per diaphragm section ± SEM; ns denotes nonsignificant changes; $^*P < .05$ and $^{***}P < .01$ versus control, as determined by Student unpaired *t* test. (E) Protein expression of Prox1, VEGFR-3, LYVE-1, NF-κB p50 phosphorylated on Ser337, nonphosphorylated NF-κB p50, NF-κB p65 phosphorylated on Ser276, nonphosphorylated NF-κB p65, and β-actin was determined by Western blot of combined lysates (100 μg of total protein per lane) derived from 3 to 4 mice per group. (F) Protein expression in Western blots was determined by band densitometry. Values were normalized to β-actin and are shown as fold increase relative to expression of corresponding proteins in untreated control mice at day 0.

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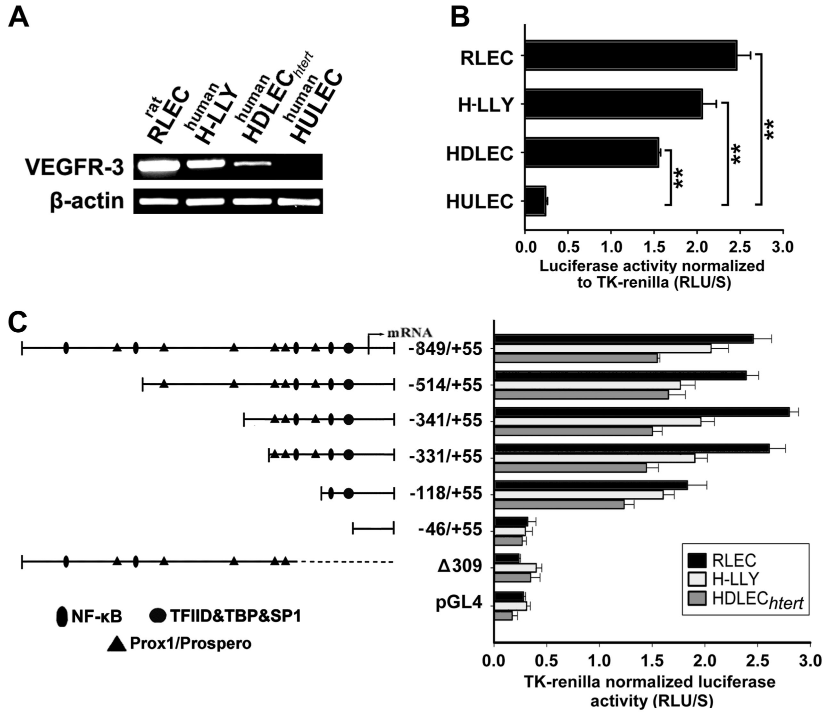

Figure 3. VEGFR-3 promoter characterization and gene expression in lymphatic endothelial cells. (A) VEGFR-3 mRNA expression and (B) full-length VEGFR-3$^{-849/+55}$ promoter activity were measured in the lymphatic endothelial cell lines RLECs, H-LLY, and HDLECs$_{htert}$. Human lung blood microvascular endothelial cell line, HULEC, was used as a VEGFR-3–negative cell line. Data shown are a representative image of VEGFR-3 transcript expression of 3 independent experiments (A) and the mean promoter activity of 3 independent experiments ± SEM (B). $^{**}P < .01$ versus VEGFR-3 promoter activity in the negative control cell line HULEC as determined by Student unpaired *t* test. (C) Activities of VEGFR-3 promoter deletion constructs were tested in RLECs, H-LLY, and HDLECs$_{htert}$. The left panel shows schematic illustration of deletion constructs with relative locations of predicted transcription factor binding sites. The right panel shows VEGFR-3 promoter activity of deletion constructs presented as relative light units per second (RLU/S) normalized per renilla luciferase activity of cotransfected thymidine kinase (TK)–renilla plasmid. Experiments were performed in duplicate and reproduced at least 3 times. Data are presented as the mean promoter activity of 3 independent experiments ± SEM.

by Prox1 up-regulation that doubled on day 1 of inflammation and nearly tripled on day 2 (Figure 2F). In contrast, the peak of VEGFR-3 expression was delayed to day 4, on which its level in inflamed tissues was 2.8-fold higher compared with controls (supplemental Table 2). Consistent with immunostaining, no changes in LYVE-1 protein were detected over 7 days of treatment. These data suggest that activation of NF-κB and Prox1 might be responsible for LEC activation, VEGFR-3 elevation, and lymphangiogenesis. Because no significant changes in LVD were detected in the first week of inflammation, these findings imply that NF-κB, Prox1, and VEGFR-3 are all required for lymphangiogenesis that is preceded by up-regulation of these proteins by 3 to 5 days.

Characterization of the human VEGFR-3 regulatory elements

To gain further insights into inflammation-dependent induction of VEGFR-3, we cloned and characterized the human VEGFR-3 promoter. Previous testing of the mouse VEGFR-3 promoter[38] demonstrated that the proximal 0.8 kb is sufficient to mediate cell type–specific transcriptional activity. However, NF-κB– and Prox1-dependent regulation of human or mouse promoter has not been previously examined.

High activity of the VEGFR-3$^{-849/+55}$ promoter was detected in 3 LEC lines with endogenous VEGFR-3 expression (Figure 3A). Promoter activity in LECs was 10.25-fold (± 0.7-fold; RLECs), 8.6-fold (± 0.7-fold; H-LLY), and 6.5-fold (± 0.2-fold; HDLECs$_{htert}$) higher than in the human blood vascular endothelial line, HULEC (Figure 3B). Promoter-reporter specificity was confirmed by empty vector and a promoter construct lacking the transcription start site (Δ309), both of which had 10% of the activity mediated by full-length VEGFR-3$^{-849/+55}$ (Figure 3C).

To identify the core elements required for transcriptional activity of the promoter we performed deletion analysis. Truncation from −849 bp to −331 bp did not significantly affect promoter activity (Figure 3C), suggesting that *cis*-acting response elements are located within the proximal −331/+55-bp region. Similar promoter activity was measured in human and rat LECs suggesting that the regulatory elements are conserved among species. Analysis of the −331/+55-bp region identified putative binding sites for several transcription factors including NF-κB and Prox1. Promoter truncation from −331 bp to −118 bp reduced activity by 15% to 30%, whereas reduction to −46 bp reduced luciferase activity to the level of control Δ309 construct (Figure 3C). This suggested that NF-κB and Prox1, whose binding sites are located within the proximal −331-bp region, are responsible for up-regulation of VEGFR-3 observed in vivo.

NF-κB transcription factors regulate VEGFR-3 expression in LECs

To determine the role of NF-κB in regulation of VEGFR-3 expression, LECs were cotransfected with the VEGFR-3$^{-849/+55}$ promoter and pCMV-Flag-p50, pCMV-Flag-p65, or empty plasmids. Equivalent expression of NF-κB subunits was determined by Western blot using Flag-specific antibody (supplemental Figure 4A). Compared with empty vector, NF-κB p65 activated VEGFR-3 promoter by 9-fold (± 1.0-fold) and 6-fold (± 0.5-fold) in H-LLY and RLECs, respectively. However, NF-κB p50 increased promoter activity by 58-fold (± 7-fold) and 51-fold (± 5-fold) in H-LLY and RLECs, respectively (Figure 4A). The difference in promoter activation was not due to functional deficiency of Flag-p65 construct as demonstrated by cotransfection with a NF-κB luciferase-reporter (supplemental Figure 4B). These data suggested that p50 has higher transactivation potential of VEGFR-3 promoter than p65 protein.

We used ChIP assay to determine whether NF-κB subunits bind the VEGFR-3 promoter. Primers were designed to encompass the region that includes or lacks the potential NF-κB sites. Only the −403/−238-bp promoter segment was detected by anti-p50, anti-p65, and anti–acetylated-H3 antibodies, indicating binding and active transcription by NF-κB in this region. ChIP analysis showed preferential binding by the p50 subunit (Figure 4B). Nonspecific rabbit IgG and primers flanking the region devoid of

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NF-κB binding sites did not amplify PCR products, demonstrating specificity of the ChIP assay.

Inflammatory stimuli induce LEC proliferation and migration via VEGFR-3 signaling

Because the VEGFR-3 promoter was activated by NF-κB factors, we reasoned that treatment of LECs with NF-κB–dependent inflammatory mediators should increase the level of VEGFR-3 transcripts. To test this hypothesis, HDLECs$_{htert}$ were stimulated with known NF-κB activators, IL-3 (10 ng/mL) or LPS (100 ng/mL), for 6 and 24 hours, followed by qRT-PCR analysis of NF-κB p50 and p65, E-selectin, LYVE-1, and VEGFR-3 (Figure 4C-F). IL-3 and LPS treatment for 6 or 24 hours activated NF-κB signaling as demonstrated by significant increases in p50, p65, and E-selectin, a known NF-κB–regulated gene (Figure 4C-D). After 6 hours of treatment with LPS and IL-3, VEGFR-3 was up-regulated by 6.2-fold (± 0.8-fold) and 4.4-fold (± 0.7-fold), respectively. After 24 hours of treatment with these stimuli, VEGFR-3 was up-regulated by 1.6-fold (± 2.4-fold) and 2.9-fold (± 0.2-fold), respectively (Figure 4E). In comparison, LYVE-1 was unchanged by IL-3 or down-regulated after 24 hours of LPS treatment (Figure 4F), attesting to the target specificity of NF-κB stimulation.

We hypothesized that IL-3– and LPS-induced VEGFR-3 would enhance LEC proliferation and migration to VEGFR-3–specific ligands, such as, VEGF-C152S.[39] To test this hypothesis, we measured proliferation and migration of RLECs stimulated by IL-3 or LPS alone or in combination with VEGF-C152S. VEGF-C152S, IL-3, or LPS significantly increased RLEC proliferation in a dose-dependent manner, with maximum increase of 2.2-, 1.8-, and 2.4-fold compared with control, respectively (Figure 4G-I). Preactivation with IL-3 or LPS followed 6 hours later by VEGF-C152S treatment significantly increased proliferation by 18% to 39% compared with individual cytokines (Figure 4J). IL-3, LPS, or VEGF-C152S also induced RLEC migration by 2.1-, 1.6-, and 1.8-fold (Figure 4K). LEC migratory response to VEGF-C152S increased up to 44% after pretreatment with IL-3 or LPS (Figure 4K). These results suggest that VEGFR-3 up-regulation by inflammatory stimuli mediating NF-κB activation enhances LEC responsiveness to VEGFR-3–specific ligands.

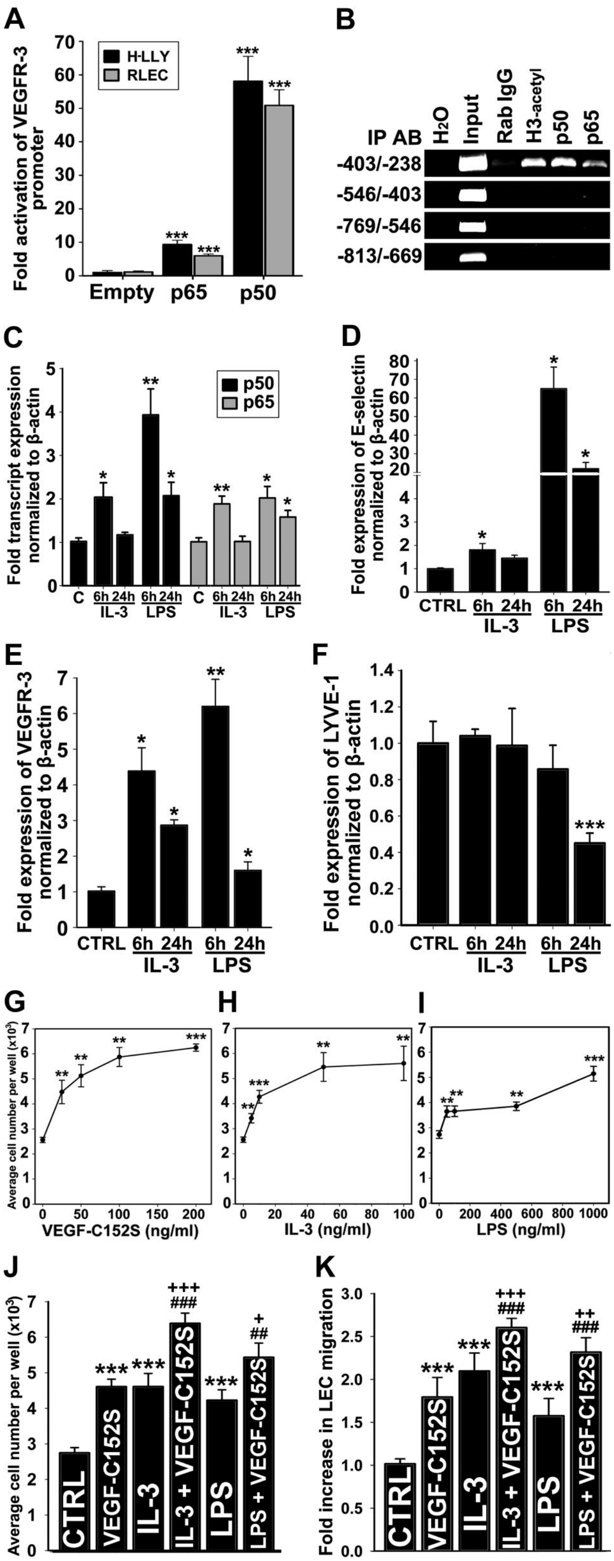

Figure 4. NF-κB pathway up-regulates VEGFR-3 expression and activates lymphatic endothelial cells. (A) VEGFR-3 promoter activity in RLECs and H-LLY cells cotransfected with VEGFR-3$^{-849/+55}$ and pCMV-Flag-p50, pCMV-Flag-p65, or empty control plasmids. Promoter activity is normalized per milligram of protein. Data presented for each cell line as the mean promoter activity ± SEM of 3 independent experiments performed in duplicate ± SEM (total n = 6 per experimental condition). ***P < .001 versus control as determined by Student unpaired t test. (B) ChIP was performed using RLECs and anti-p65, -p50, and -acetylated histone H3 antibodies (positive control), or nonspecific rabbit IgG (negative control). Immunoprecipitated chromatin was visualized by PCR using primers either flanking (−403/−238 bp) or upstream of putative NF-κB binding sites (−813/−403 bp). Data are representative of 4 independent ChIP experiments with similar results. (C-F) qRT-PCR analysis of NF-κB p50 and p65 (C), E-selectin (D), VEGFR-3 (E), and LYVE-1 (F) mRNA expression in HDLECs$_{htert}$ treated with IL-3 (10 ng/mL) or LPS (100 ng/mL) for 6 or 24 hours. The relative expression of each target was normalized to β-actin. Data are presented as the mean values of 3 independent experiments ± SEM. *P < .05, **P < .01, and ***P < .001 versus control as determined by Student unpaired t test. (G-I) RLEC proliferation induced by 72-hour exposure to VEGF-C152S (25-200 ng/mL; G), IL-3 (5-100 ng/mL; H), and LPS (50-1000 ng/mL; I). (J) Additive proliferative effects of RLECs treated with VEGF-C152S (100 ng/mL), IL-3 (10 ng/mL), or LPS (500 ng/mL) alone compared with pretreatment with IL-3 (10 ng/mL) or LPS (500 ng/mL) followed by stimulation with VEGF-C152S (100 ng/mL). (G-J) Data are presented as the average cell number of 3 independent experiments ± SEM (total n = 6 per condition). (K) Migration of RLECs induced by treatment with VEGF-C152S (200 ng/mL), IL-3 (10 ng/mL), or LPS (500 ng/mL) and combined treatment with IL-3 (10 ng/mL) and VEGF-C152S (200 ng/mL) or LPS (500 ng/mL) and VEGF-C152S (200 ng/mL). RLEC migration toward 0.25% FBS was used as a negative control. Data presented as average fold increase in RLEC migration ± SEM of 3 independent experiments. (J-K) *P < .05, **P < .01, and ***P < .001 versus control. ##P < .01 and ###P < .001 versus cytokine treatment alone. +P < .05, ++P < .01, and +++P < .001 versus VEGF-C152S treatment alone. All statistical tests were done by Student unpaired t test.

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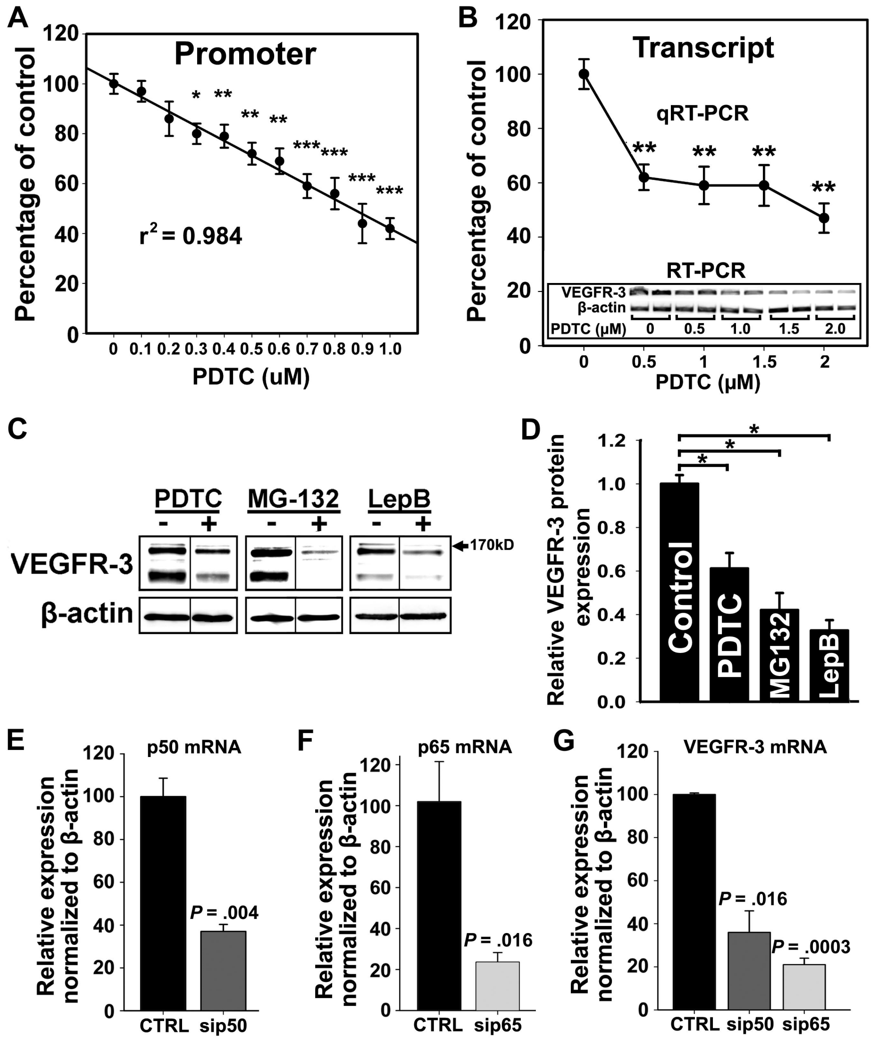

Figure 5. NF-κB signaling is required for VEGFR-3 expression in lymphatic endothelial cells. (A) RLECs were transfected with the full-length VEGFR-3$^{-849/+55}$ promoter and treated with PDTC (0-1μM) or vehicle for 18 hours. Promoter activity was measured by luciferase assay and normalized to total protein per well. Note linear inhibition of VEGFR-3 promoter activity by PDTC determined by linear regression (r^2 shown on graph) of the mean promoter activity ± SEM of 3 independent experiments performed in duplicate (total n = 6 per condition). (B) VEGFR-3 transcript expression assayed by qRT-PCR in RLECs treated with PDTC (0-2μM) or vehicle. Data are presented as mean transcript expression normalized to β-actin of 3 independent experiments ± SEM (total n = 3 per condition). Inset shows a dose-dependent decrease of VEGFR-3 transcript detected by RT-PCR. (B-C) **P* < .05 versus control, ***P* < .01 versus control, ****P* < .001 versus control, by Student unpaired *t* test. (C) Western blot analysis of RLECs treated with PDTC (7.5μM), MG-132 (0.25μM), leptomycin B (10nM), or vehicle for 24 hours. β-Actin was used as a loading control. Vertical lines have been inserted to indicate repositioned gel lanes from blots presented in supplemental Figure 6, which show dose-dependent responses to NF-κB inhibitors. (D) Densitometric values demonstrate a statistically significant decrease in VEGFR-3 protein normalized to β-actin from RLECs treated with NF-κB inhibitors or vehicle for 24 hours. Experiments were performed in duplicate and data are presented as mean normalized per β-actin VEGFR-3 expression ± SEM; **P* < .05 versus control, by Student unpaired *t* test. (E-G) H-LLY cells were transfected with p50- or p65-specific siRNA or scramble control siRNA for 48 hours and transcript expression for p50 (E), p65 (F), and VEGFR-3 (G) was determined by qRT-PCR. Data are presented as the mean transcript expression normalized to β-actin of 3 independent samples ± SEM (n = 3 per condition). Statistically significant differences were determined versus control, by Student unpaired *t* test. *P* values are displayed on the graphs.

Inhibition of NF-κB signaling represses VEGFR-3 expression in LECs

Because VEGFR-3 was elevated in inflamed lymphatic vessels (Figures 1-2) and upon forced expression of NF-κB proteins (Figure 4), we hypothesized that endogenous VEGFR-3 expression in LECs is maintained by constitutive NF-κB signaling. To test this hypothesis, we determined the effects of an NF-κB inhibitor PDTC[40] on VEGFR-3 expression at promoter, mRNA, and protein levels. PDTC-treated LECs demonstrated a dose-dependent reduction (up to 60%) of VEGFR-3 promoter activity and mRNA (Figure 5A-B). Constitutive expression and nuclear localization of p50 and p65 were also inhibited by PDTC, which coincided with decreased VEGFR-3 expression (supplemental Figure 5). Neither cell viability (supplemental Figure 6) nor expression of NF-κB–independent targets (eg, β-actin) was affected by PDTC at the tested concentrations (Figure 5B inset). This effect was reproduced by 2 other inhibitors: MG-132, a blocker of IκB-α degradation,[40] and leptomycin B, an inhibitor of NF-κB nuclear transport.[41] Western blot showed up to 70% reduction of VEGFR-3 expression by all inhibitors in a dose-dependent manner (Figure 5C-D). Drug concentrations that repressed VEGFR-3 protein expression were at least 10-fold below the median inhibitory concentration values for LECs (supplemental Figure 6).

NF-κB regulation of VEGFR-3 expression was also confirmed by target-specific knockdown of NF-κB subunits. H-LLY cells were transfected with siRNA targeting p50 or p65 or scrambled control siRNA. qRT-PCR performed 48 hours after transfection showed 50% to 70% knockdown of p50 and p65 (Figure 5E-F) and a corresponding 50% to 80% reduction in VEGFR-3 transcripts (Figure 5G). Collectively, these data suggest that NF-κB is involved in regulation of endogenous VEGFR-3 expression.

The VEGFR-3 promoter is activated by Prox1

Prox1 has been reported to induce VEGFR-3 expression in cultured endothelial cells.[18,20] However, Prox1 regulates more than 90 genes[20] and transactivation of the VEGFR-3 promoter by Prox1 has not been previously shown. To determine whether Prox1 transcriptionally regulates VEGFR-3, LECs were cotransfected with VEGFR-3$^{-849/+55}$ promoter and escalating concentrations (0-0.5 μg) of a Prox1-encoding or empty vector followed by measurement of luciferase activity. Relative to empty-vector control, overexpression of Prox1 increased VEGFR-3$^{-849/+55}$ activity in a linear dose-dependent manner by 76-fold and 7-fold in H-LLY and RLECs, respectively (Figure 6A-B).

Several putative Prox1 binding sites, analogous to published consensus sequences (CA/tc/tNNCT/c and TA/tAGNC/tN[42]), are present in both human and rat VEGFR-3 promoters. ChIP assay in RLECs showed that the region containing consensus Prox1 binding sites (−403/−238 bp) was immunoprecipitated by anti-Prox1 antibody (Figure 6C, supplemental Figure 7). Prox1 antibodies did

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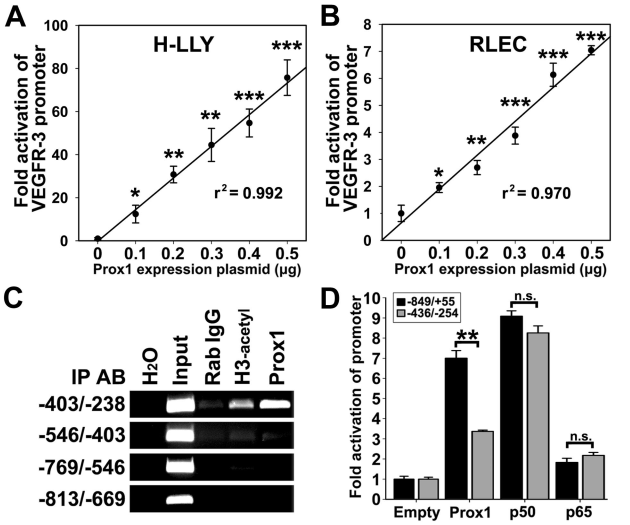

Figure 6. Prox1 directly activates the VEGFR-3 promoter. VEGFR-$3^{-849/+55}$ promoter plasmid was cotransfected with pCMV-Prox1 plasmid (0-0.5 μg) in H-LLY cells (A) and RLECs (B). Promoter activity was measured by luciferase assay and normalized per milligram of protein. Note linear response to Prox1 transactivation in both cell lines as determined by linear regression (r^2 shown on graph) of the mean promoter activity ± SEM of 3 independent experiments performed in duplicate (n = 6 per condition; A-B). (A-B) **P* < .05 versus control, ***P* < .01 versus control, ****P* < .001 versus control, by Student unpaired *t* test. (C) ChIP analysis of the VEGFR-3 promoter was performed on RLECs as described in the legend for Figure 4. Immunoprecipitated chromatin was visualized by PCR with primers flanking transcription factor binding sites (−403/−238 bp) or upstream of binding sites (−813/−403 bp). Data are representative of 3 independent ChIP experiments with similar results. (D) Fold activation of a truncated VEGFR-3 promoter (−436/−254) was compared with the full-length VEGFR-$3^{-849/+55}$. RLECs were cotransfected with 0.5 μg of VEGFR-$3^{-849/+55}$ or VEGFR-$3^{-436/-254}$ promoter plasmids and 0.5 μg of pCMV-Prox1, pCMV-Flag-p50, pCMV-Flag-p65, or empty control plasmid. Promoter activity is normalized per milligram of protein. Data are presented as the mean promoter activity of 3 independent experiments performed in duplicate ± SEM (total n = 6 per experimental condition). ns denotes nonsignificant changes. ***P* < .01 versus control as determined by Student unpaired *t* test.

not pull down other flanking promoter DNA, indicating specific interaction between Prox1 and the VEGFR-3 promoter within a promoter segment that was also bound by NF-κB (Figure 4B). To test whether this region is crucial for promoter activation, RLECs were cotransfected with a construct encoding bases −436 to −254 (VEGFR-$3^{-436/-254}$) and pCMV-Prox1, pCMV-Flag-p50, pCMV-Flag-p65, or empty plasmids. VEGFR-$3^{-849/+55}$ and VEGFR-$3^{-436/-254}$ were identically activated by p50 and p65, whereas Prox1 fold activation of VEGFR-$3^{-436/-254}$ was reduced approximately by half compared with the full-length promoter (Figure 6D). This suggests that full activation by Prox1 might require interaction with additional sites outside of the −436/−254-bp region.

NF-κB regulates Prox1 expression in LECs

We found that forced expression of Prox1 activated VEGFR-3 promoter in vitro and both Prox1 and VEGFR-3 are induced by inflammation in vivo with Prox1 up-regulation preceding that of VEGFR-3 (Figure 2). This suggested that NF-κB might first up-regulate Prox1 followed by cooperative regulation of VEGFR-3. To test this hypothesis, the level of Prox1 expression was quantified by qRT-PCR after 6-hour stimulation by IL-3 (10 ng/mL), conditions that increased VEGFR-3 expression (Figure 4E). IL-3 significantly increased Prox1 by 2-fold ($P < .01$, Figure 7A), implicating Prox1 as a downstream target of NF-κB.

We next investigated the effects of NF-κB inhibitors on Prox1 expression. PDTC suppressed Prox1 mRNA by approximately 60% (Figure 7B), suggesting that Prox1 transcription requires NF-κB. Western blot showed that PDTC, MG-132, and leptomycin B all significantly repressed Prox1 expression (Figure 7C). Moreover, p50 and p65 siRNA but not scrambled control also decreased Prox1 expression by 60% (Figure 7D), corroborating the hypothesis that NF-κB regulates Prox1 in LECs.

Prox1 and NF-κB synergistically activate the VEGFR-3 promoter

Cultured LECs express high levels of Prox1 and p50, making it difficult to evaluate the contributions of these factors to VEGFR-3 transcription. To test whether Prox1 and NF-κB cooperate in activation of the VEGFR-3 promoter, we used Prox1-negative nonendothelial (HEK293) and endothelial (HULEC) lines. Similar results were obtained in both lines cotransfected with Prox1 (Figure 7E-F insets), the full-length VEGFR-$3^{-849/+55}$ promoter and Flag-p50, Flag-p65, or empty vector. In the absence of Prox1, p50 weakly activated the VEGFR-3 promoter, whereas p65 had no effect. Prox1 combined with p65 did not increase promoter activity compared with Prox1 alone. In contrast, Prox1 combined with p50 activated the VEGFR-3 promoter 22.3-fold (± 0.7-fold) and 66.9-fold (± 3.8-fold) over the vector control in HEK293 cells and HULECs, respectively (Figure 7E-F). Combination of these plasmids had no effect on the activity of NF-κB–independent ubiquitin C (UBC) or phosphoglycerate kinase (PGK) promoters. The activity of a truncated VEGFR-$3^{-118/+55}$ promoter in response to Prox1, p50, and p65 alone or in combination was significantly reduced compared with the responses of the full-length VEGFR-$3^{-849/+55}$ (Figure 7G, supplemental Figure 8). These results confirm the functionality of the region beyond −118 bp and suggest that Prox1 and NF-κB p50 synergistically activate the VEGFR-3 promoter.

Discussion

Inflammation and NF-κB signaling up-regulate VEGFR-3 expression during lymphangiogenesis

Inflammation is the main physiologic event that evokes formation of new lymphatic vessels in adulthood.[43] Although the role of inflammation in induction of lymphangiogenesis has long been recognized, the underlying molecular mechanisms remained undefined. We present novel evidence that inflammation-induced NF-κB signaling precedes lymphatic-specific up-regulation of VEGFR-3 and that NF-κB activates VEGFR-3 transcription in cultured LECs (Figures 1-2,4). Moreover, our data show that NF-κB–dependent mediators, IL-3 and LPS, increase VEGFR-3 expression and responsiveness of LECs to VEGFR-3–activating factors (Figure 4). Collectively, these results suggest that LEC stimulation by NF-κB–dependent cytokines amplifies the lymphangiogenic signals by increasing VEGFR-3 expression.

In vivo analysis demonstrated that up-regulation of VEGFR-3 on preexisting vessels preceded formation of new LYVE-1^+ vessels by several days (Figures 1-2), suggesting that elevated VEGFR-3 expression is crucial for induction of lymphangiogenesis. This is consistent with previous reports demonstrating the paramount role of VEGFR-3 for LEC activation and inflammatory lymphangiogenesis as shown by blocking this receptor in models of LPS-induced peritonitis,[44] chronic airway infection,[9] wound healing,[16] and cancer.[17] VEGFR-3 ligands, VEGF-C/-D, are highly expressed during inflammation by infiltrating immune cells, such as CD11b$^+$ macrophages.[44,45] The abundant expression of VEGF-C/-D at inflamed sites suggests that lymphangiogenesis might be restricted by limited VEGFR-3 expression on preexisting

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lymphatic vessels rather than by ligand availability. A low density of VEGFR-3 receptors may result in self-limiting lymphangiogenesis due to receptor saturation and internalization. In contrast, high level of VEGFR-3 expression might be induced by inflammation due to sustained cytokine production and continuous activation of NF-κB in LECs.

Prox1 is up-regulated during inflammation and mediates VEGFR-3 expression

Prox1 is an essential mediator of embryonic lymphangiogenesis,[18,20] but little is known about its functions in adulthood. We present novel evidence that Prox1 expression is rapidly increased after the onset of inflammation preceding both VEGFR-3 up-regulation and lymphangiogenesis (Figures 1-2). We also showed that the NF-κB–dependent cytokine, IL-3, up-regulates Prox1 in adult LECs, which is consistent with prior reports that IL-3 induced Prox1 expression in adult BECs.[22] Elevated expression of Prox1 and VEGFR-3 has also been shown in Kaposi sarcoma.[46] However, the latter finding could be construed as induction by tumor-derived factors rather than by sustained NF-κB activation. In comparison, we report here lymphatic-specific induction of Prox1 by NF-κB–dependent cytokines and suppression of Prox1 by NF-κB–specific inhibitors. These data suggest that Prox1 might regulate responsiveness to inflammation in adult LECs.

Prox1 regulates acquisition of lymphatic phenotype during embryogenesis[18] and transdifferentiation of adult BECs to LECs.[18,20] Up-regulation of Prox1 induces VEGFR-3,[18,20] whereas silencing Prox1 suppresses VEGFR-3 expression.[21] Our data suggest that Prox1 induces VEGFR-3 expression through promoter transactivation as indicated by ChIP and a dose-dependent increase in promoter activity (Figure 6). These data identify Prox1 as a potential downstream target of NF-κB and a regulator of VEGFR-3 expression under inflammatory conditions.

VEGFR-3 transcription is predominantly regulated by NF-κB p50 that might cooperate with Prox1

We show that NF-κB p50, rather than p65, is the predominant activator of VEGFR-3 (Figures 4,7). Preferential activation by p50 was reported for other promoters including Bcl-2[47] and PDGF-A.[48] In contrast, promoters of some NF-κB responsive genes (eg, TNF-α and IL-8) are suppressed by p50 homodimers.[49] This suggests that p50 can function as a transcriptional activator or repressor depending on cellular context, sequence of the response element, and transcriptional cofactors. Because p50 lacks a consensus transactivation domain,[24] p50-driven transcription requires coactivators, such as Bcl-3[47] or C/EBP proteins,[50] that might be present in LECs. Both Prox1 and p50 have been shown to interact with the transcriptional coactivator, CBP/p300,[50] suggesting that such protein-protein interaction might account for synergistic activation of the VEGFR-3 promoter (Figure 7). Prox1 might also

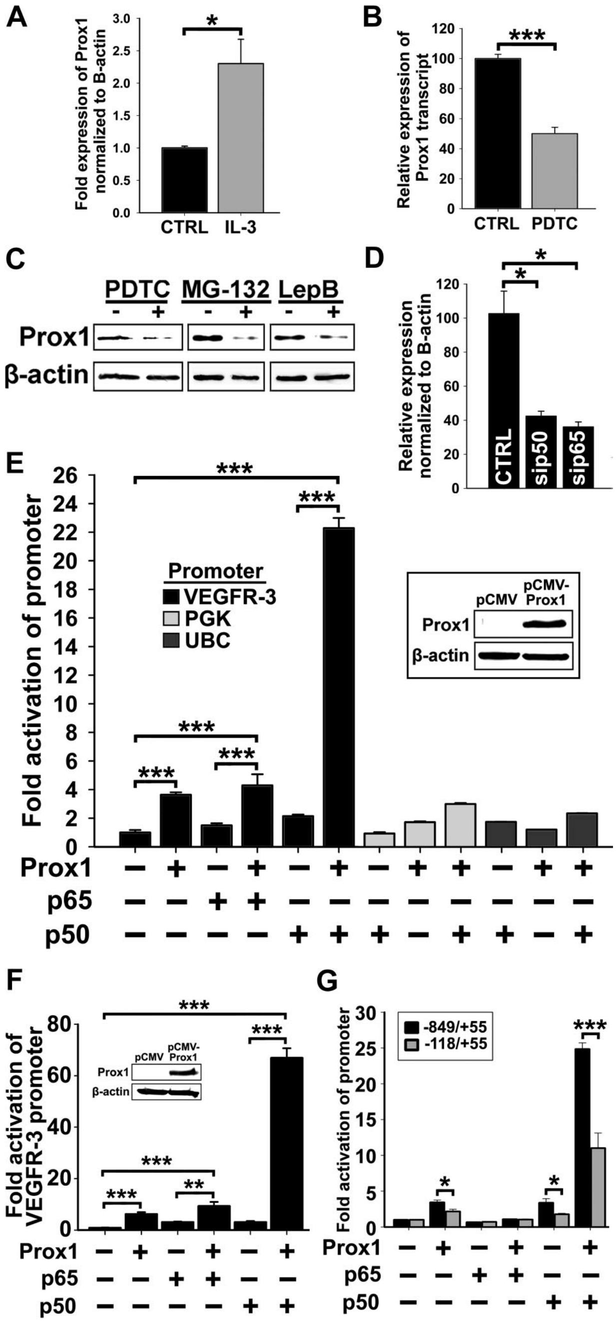

Figure 7. The p50 subunit of NF-κB up-regulates Prox1, and both p50 and Prox1 synergistically regulate VEGFR-3 expression. (A) qRT-PCR analysis of Prox1 transcripts in HDLECs$_{htert}$ treated with IL-3 (10 ng/mL) for 6 hours. (B) qRT-PCR analysis of Prox1 transcripts in RLECs treated with PDTC (2.5μM) for 24 hours. (A-B) Data are presented as β-actin normalized mean transcript expression of 3 independent experiments performed in duplicate ± SEM (total: n = 6 per condition). (C) Prox1 detected by Western blot of nuclear extracts from RLECs treated with PDTC (5μM), MG-132 (250nM), leptomycin B (10nM), or vehicle alone. β-Actin was used as a loading control. Representative data are shown from 1 of 3 experiments. (D) qRT-PCR analysis of Prox1 transcript in H-LLY transfected with p50 and p65 siRNA. Data are presented as the mean transcript expression normalized to β-actin ± SEM derived from 3 independent samples. (E) Prox1-negative nonendothelial line HEK293 was transfected with VEGFR-3$^{-849/+55}$ promoter plasmid and pCMV-Flag-p50 or pCMV-Flag-p65 plasmids and cotransfected with pCMV-Prox1 or empty vector (0.25 μg of each plasmid). VEGFR-3 promoter activity was normalized to total milligram of protein. Inset confirms lack of Prox1 in control HEK293 and forced expression in transfected cells. Activation of VEGFR-3 promoter by coexpression of p50 and Prox1 was compared with the effect on NF-κB–independent promoters for phosphoglycerate kinase (PGK) and ubiquitin C (UBC) examined under the same conditions. Data presented as the mean promoter activity ± SEM of 3 independent experiments performed in triplicate (total n = 9 per condition). (F) Prox1-negative blood vascular endothelial line, HULEC, was transfected with VEGFR-3$^{-849/+55}$ promoter expression and pCMV-Flag-p50 or pCMV-Flag-p65 plasmids and cotransfected with pCMV-Prox1 or empty vector, as described in panel E. The analysis of the VEGFR-3 promoter activity was performed as described in panel E. Data are presented as the mean VEGFR-3 promoter activity ± SEM derived from 3 independent experiments performed in quadruplicate (total n = 12 per condition). (G) Fold activation of the full-length (−849/+55 bp) and truncated (−118/+55 bp) VEGFR-3 promoters was compared after cotransfection with pCMV-Prox1, pCMV-Flag-p50, and pCMV-Flag-p65 alone or in combination as described in panel E. Data are presented as the mean VEGFR-3 promoter activity ± SEM derived from 3 independent experiments. *$P < .05$, **$P < .01$, and ***$P < .001$ versus control, as determined by Student unpaired *t* test.

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be a lymphatic-specific coactivator of p50, which would account for the weak transactivation of the VEGFR-3 promoter by p50 in Prox1-negative BECs (Figure 7) and the lack of VEGFR-3 up-regulation on inflamed blood vasculature (supplemental Figure 3). This is consistent with overlapping peaks and protein kinetics of p-p50 and Prox1 observed in vivo (Figure 2), suggesting that Prox1 might confer lymphatic specificity to ubiquitously activated NF-κB signaling during inflammation. Thus, the cooperative role of Prox1 with p50 in regulation of VEGFR-3 transcription could be 2-fold: to amplify p50 signaling and to impart lymphatic specificity to activated NF-κB, promoting lymphangiogenesis-required gene expression.

In summary, we demonstrate that increased Prox1 and VEGFR-3 expression precedes lymphangiogenesis in vivo. Increased expression of VEGFR-3 is likely mediated by Prox1 and NF-κB binding to its promoter. Prox1 induced by NF-κB synergizes with p50 in activation of the promoter, suggesting a complex interplay between ubiquitous and lymphatic-specific proteins. Future delineation of these mechanisms might identify targets for therapeutic control of abnormal lymphangiogenesis induced at chronically inflamed sites and malignancy.

Acknowledgments

We thank Dr Albert Baldwin and Stephen Markwell for providing NF-κB plasmids and statistical data analysis, respectively. We also thank Lisa Volk and Kathleen Brancato for assistance with animal experiments and immunostaining, respectively.

This study was funded, in part, by the National Institutes of Health (no. 1R15CA125682-01), Illinois William E. McElroy Foundation and SIUSOM Excellence in Medicine awards (S.R.), and by a Department of Defense Breast Cancer Research Program predoctoral traineeship (no. BC073318; M.J.F.).

Authorship

Contribution: M.J.F. designed research, performed or supervised experiments, analyzed and interpreted data, and wrote the paper; A.W. contributed vital new reagents and wrote the paper; K.L.H. performed siRNA experiments; C.I. and K.M. contributed vital new reagents; R.E.N. and M.S.P. contributed HDLECs$_{htert}$; D.C.Z. contributed RLECs; and S.R. designed research, supervised experiments, analyzed and interpreted data, and wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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References

1. Swartz MA, Hubbell JA, Reddy ST. Lymphatic drainage function and its immunological implications: from dendritic cell homing to vaccine design. *Semin Immunol.* 2008;20(2):147-156.
2. Shin WS, Rockson SG. Animal models for the molecular and mechanistic study of lymphatic biology and disease. *Ann N Y Acad Sci.* 2008; 1131:50-74.
3. Jamieson T, Cook DN, Nibbs RJ, et al. The chemokine receptor D6 limits the inflammatory response in vivo. *Nat Immunol.* 2005;6(4):403-411.
4. Achen MG, Stacker SA. Molecular control of lymphatic metastasis. *Ann N Y Acad Sci.* 2008;1131: 225-234.
5. Veikkola T, Jussila L, Makinen T, et al. Signalling via vascular endothelial growth factor receptor-3 is sufficient for lymphangiogenesis in transgenic mice. *EMBO J.* 2001;20(6):1223-1231.
6. Kaipainen A, Korhonen J, Mustonen T, et al. Expression of the fms-like tyrosine kinase 4 gene becomes restricted to lymphatic endothelium during development. *Proc Natl Acad Sci U S A.* 1995;92(8):3566-3570.
7. Whitehurst B, Flister MJ, Bagaitkar J, et al. Anti-VEGF-A therapy reduces lymphatic vessel density and expression of VEGFR-3 in an orthotopic breast tumor model. *Int J Cancer.* 2007;121(10): 2181-2191.
8. Paavonen K, Puolakkainen P, Jussila L, Jahkola T, Alitalo K. Vascular endothelial growth factor receptor-3 in lymphangiogenesis in wound healing. *Am J Pathol.* 2000;156(5):1499-1504.
9. Baluk P, Tammela T, Ator E, et al. Pathogenesis of persistent lymphatic vessel hyperplasia in chronic airway inflammation. *J Clin Invest.* 2005; 115(2):247-257.
10. Kunstfeld R, Hirakawa S, Hong YK, et al. Induction of cutaneous delayed-type hypersensitivity reactions in VEGF-A transgenic mice results in chronic skin inflammation associated with persistent lymphatic hyperplasia. *Blood.* 2004;104(4): 1048-1057.
11. Zhang Q, Lu Y, Proulx ST, et al. Increased lymphangiogenesis in joints of mice with inflammatory arthritis. *Arthritis Res Ther.* (http://arthritis-research.com/content/pdf/ar2326.pdf). 2007;9(6): R118.
12. Maruyama K, Ii M, Cursiefen C, et al. Inflammation-induced lymphangiogenesis in the cornea arises from CD11b-positive macrophages. *J Clin Invest.* 2005;115(9):2363-2372.
13. Cursiefen C, Chen L, Borges LP, et al. VEGF-A stimulates lymphangiogenesis and hemangiogenesis in inflammatory neovascularization via macrophage recruitment. *J Clin Invest.* 2004; 113(7):1040-1050.
14. Iwata C, Kano MR, Komuro A, et al. Inhibition of cyclooxygenase-2 suppresses lymph node metastasis via reduction of lymphangiogenesis. *Cancer Res.* 2007;67(21):10181-10189.
15. Ristimäki A, Narko K, Enholm B, Joukov V, Alitalo K. Proinflammatory cytokines regulate expression of the lymphatic endothelial mitogen vascular endothelial growth factor-C. *J Biol Chem.* 1998; 273(14):8413-8418.
16. Goldman J, Rutkowski JM, Shields JD, et al. Cooperative and redundant roles of VEGFR-2 and VEGFR-3 signaling in adult lymphangiogenesis. *FASEB J.* 2007;21(4):1003-1012.
17. Roberts N, Kloos B, Cassella M, et al. Inhibition of VEGFR-3 activation with the antagonistic antibody more potently suppresses lymph node and distant metastases than inactivation of VEGFR-2. *Cancer Res.* 2006;66(5):2650-2657.
18. Hong YK, Harvey N, Noh YH, et al. Prox1 is a master control gene in the program specifying lymphatic endothelial cell fate. *Dev Dyn.* 2002; 225(3):351-357.
19. Wigle JT, Harvey N, Detmar M, et al. An essential role for Prox1 in the induction of the lymphatic endothelial cell phenotype. *EMBO J.* 2002;21(7): 1505-1513.
20. Petrova TV, Makinen T, Makela TP, et al. Lymphatic endothelial reprogramming of vascular endothelial cells by the Prox-1 homeobox transcription factor. *EMBO J.* 2002;21(17):4593-4599.
21. Mishima K, Watabe T, Saito A, et al. Prox1 induces lymphatic endothelial differentiation via integrin alpha9 and other signaling cascades. *Mol Biol Cell.* 2007;18(4):1421-1429.
22. Gröger M, Loewe R, Holnthoner W, et al. IL-3 induces expression of lymphatic markers Prox-1 and podoplanin in human endothelial cells. *J Immunol.* 2004;173(12):7161-7169.
23. Karin M. Nuclear factor-kappaB in cancer development and progression. *Nature.* 2006;441(7092):431-436.
24. Beinke S, Ley SC. Functions of NF-kappaB1 and NF-kappaB2 in immune cell biology. *Biochem J.* 2004;382(pt 2):393-409.
25. Kiriakidis S, Andreakos E, Monaco C, et al. VEGF expression in human macrophages is NF-kappaB-dependent: studies using adenoviruses expressing the endogenous NF-kappaB inhibitor IkappaBalpha and a kinase-defective form of the IkappaB kinase 2. *J Cell Sci.* 2003; 116(pt 4):665-674.
26. Tsai PW, Shiah SG, Lin MT, Wu CW, Kuo ML. Up-regulation of vascular endothelial growth factor C in breast cancer cells by heregulin-beta 1: a critical role of p38/nuclear factor-kappa B signaling pathway. *J Biol Chem.* 2003;278(8):5750-5759.
27. Wilber A, Frandsen JL, Wangensteen KJ, et al. Dynamic gene expression after systemic delivery of plasmid DNA as determined by in vivo bioluminescence imaging. *Hum Gene Ther.* 2005;16(11): 1325-1332.
28. Whitehurst B, Eversgerd C, Flister M, et al. Molecular profile and proliferative responses of rat lymphatic endothelial cells in culture. *Lymphat Res Biol.* 2006;4(3):119-142.
29. Nisato RE, Harrison JA, Buser R, et al. Generation and characterization of telomerase-transfected human lymphatic endothelial cells with an extended life span. *Am J Pathol.* 2004; 165(1):11-24.
30. Tammela T, Saaristo A, Lohela M, et al. Angiopoietin-1 promotes lymphatic sprouting and hyperplasia. *Blood.* 2005;105(12):4642-4648.
31. National Center for Biotechnology Information. GenBank. http://www.ncbi.nlm.nih.gov/Genbank. Accessed February 14, 2007.

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32. Genomatix. MatInspector. http://www.genomatix.de/products/MatInspector/index.html. Accessed March 02, 2007.
33. Laderach D, Compagno D, Danos O, Vainchenker W, Galy A. RNA interference shows critical requirement for NF-kappa B p50 in the production of IL-12 by human dendritic cells. *J Immunol.* 2003;171(4):1750-1757.
34. Surabhi RM, Gaynor RB. RNA interference directed against viral and cellular targets inhibits human immunodeficiency virus type 1 replication. *J Virol.* 2002;76(24):12963-12973.
35. Kilic N, Oliveira-Ferrer L, Neshat-Vahid S, et al. Lymphatic reprogramming of microvascular endothelial cells by CEA-related cell adhesion molecule-1 via interaction with VEGFR-3 and Prox1. *Blood.* 2007;110(13):4223-4233.
36. Oka M, Iwata C, Suzuki HI, et al. Inhibition of endogenous TGF-{beta} signaling enhances lymphangiogenesis. *Blood.* 2008;111(9):4571-4579.
37. Srinivasan RS, Dillard ME, Lagutin OV, et al. Lineage tracing demonstrates the venous origin of the mammalian lymphatic vasculature. *Genes Dev.* 2007;21(19):2422-2432.
38. Iljin K, Karkkainen MJ, Lawrence EC, et al. VEGFR3 gene structure, regulatory region, and sequence polymorphisms. *FASEB J.* 2001;15(6):1028-1036.
39. Kirkin V, Mazitschek R, Krishnan J, et al. Characterization of indolinones which preferentially inhibit VEGF-C- and VEGF-D-induced activation of VEGFR-3 rather than VEGFR-2. *Eur J Biochem.* 2001;268(21):5530-5540.
40. Dai Y, Rahmani M, Grant S. Proteasome inhibitors potentiate leukemic cell apoptosis induced by the cyclin-dependent kinase inhibitor flavopiridol through a SAPK/JNK- and NF-kappaB-dependent process. *Oncogene.* 2003;22(46):7108-7122.
41. Walsh MD Jr, Hamiel CR, Banerjee A, et al. Exportin 1 inhibition attenuates nuclear factor-kappaB-dependent gene expression. *Shock.* 2008;29(2):160-166.
42. Chen X, Taube JR, Simirskii VI, Patel TP, Duncan MK. Dual roles for Prox1 in the regulation of the chicken betaB1-crystallin promoter. *Invest Ophthalmol Vis Sci.* 2008;49(4):1542-1552.
43. Mouta C, Heroult M. Inflammatory triggers of lymphangiogenesis. *Lymphat Res Biol.* 2003;1(3):201-218.
44. Kataru RP, Jung K, Jang C, et al. Critical role of CD11b+ macrophages and VEGF in inflammatory lymphangiogenesis, antigen clearance, and inflammation resolution. *Blood.* 2009;113(22):5650-5659.
45. Kang S, Lee SP, Kim KE, et al. Toll-like receptor 4 in lymphatic endothelial cells contributes to LPS-induced lymphangiogenesis by chemotactic recruitment of macrophages. *Blood.* 2009;113(11):2605-2613.
46. Hong YK, Foreman K, Shin JW, et al. Lymphatic reprogramming of blood vascular endothelium by Kaposi sarcoma-associated herpesvirus. *Nat Genet.* 2004;36(7):683-685.
47. Kurland JF, Kodym R, Story MD, et al. NF-kappaB1 (p50) homodimers contribute to transcription of the bcl-2 oncogene. *J Biol Chem.* 2001;276(48):45380-45386.
48. Aizawa K, Suzuki T, Kada N, et al. Regulation of platelet-derived growth factor-A chain by Kruppel-like factor 5: new pathway of cooperative activation with nuclear factor-kappaB. *J Biol Chem.* 2004;279(1):70-76.
49. Tong X, Yin L, Washington R, Rosenberg DW, Giardina C. The p50-p50 NF-kappaB complex as a stimulus-specific repressor of gene activation. *Mol Cell Biochem.* 2004;265(1-2):171-183.
50. Chen Q, Dowhan DH, Liang D, Moore DD, Overbeek PA. CREB-binding protein/p300 co-activation of crystallin gene expression. *J Biol Chem.* 2002;277(27):24081-24089.

Appendix B

DOI:10.1111/j.1549-8719.2010.00057.x

Original Article

Characterization of Prox1 and VEGFR-3 Expression and Lymphatic Phenotype in Normal Organs of Mice Lacking p50 Subunit of NF-κB

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Received 7 July 2010; accepted 19 August 2010.

ABSTRACT

Objective: Inflammation and nuclear factor-kappa B (NF-κB) are highly associated with lymphangiogenesis but the underlying mechanisms remain unclear. We recently established that activated NF-κB p50 subunit increases expression of the main lymphangiogenic mediators, vascular endothelial growth factor receptor-3 (VEGFR-3) and its transcriptional activator, Prox1. To elucidate the role of p50 in lymphatic vasculature, we compared lymphatic vessel density (LVD) and phenotype in p50 knockout (KO) and wild-type (WT) mice.

Methods: Normal tissues from KO and WT mice were stained for lymphatic vessel endothelial hyaluronan receptor-1 to calculate LVD. VEGFR-3 and Prox1 expressions were analyzed by immunofluorescence and qRT-PCR.

Results: Compared with WT, LVD in the liver and lungs of KO mice was reduced by 39% and 13%, respectively. This corresponded to 25–44% decreased VEGFR-3 and Prox1 expression. In the mammary fat pad (MFP), LVD was decreased by 18% but VEGFR-3 and Prox1 expression was 80–140% higher than in WT. Analysis of p65 and p52 NF-κB subunits and an array of inflammatory mediators showed a significant increase in p50 alternative pathways in the MFP but not in other organs.

Conclusions: These findings demonstrate the role of NF-κB p50 in regulating the expression of VEGFR-3, Prox1 and LVD in the mammary tissue, liver, and lung.

Key words: lymphatic vessels, nuclear factor-kappa B, p50, vascular endothelial growth factor receptor-3, Prox1

Please cite this paper as: Flister, Volk and Ran (2010). Characterization of Prox1 and VEGFR-3 Expression and Lymphatic Phenotype in Normal Organs of Mice Lacking p50 Subunit of NF-κB. *Microcirculation* **00**, 00–00.

INTRODUCTION

The function of lymphatic vessels is important for proper immune cell transport [35], response to injury [55], homeostasis [53] and dissemination of tumor cells [28,61]. The formation of new lymphatic vessels, a process known as lymphangiogenesis, is a frequent event during embryogenesis but it is tightly regulated in adulthood. The key protein that mediates lymphangiogenesis is the vascular endothelial growth factor receptor (VEGFR)-3 [74]. This protein is expressed primarily on lymphatic endothelial cells (LECs) and is activated by growth factors VEGF-C and VEGF-D [3]. Activation of VEGFR-3 signaling increases LEC proliferation, migration, and survival [46,74], whereas blocking the VEGFR-3 pathway inhibits both inflammation-induced [5] and cancer-promoted [63] lymphangiogenesis.

Typically, acute inflammation does not elicit pro-lymphangiogenic response. However, chronic inflammation frequently induces new lymphatic vessels as has been described in malignancy [28,61] and those associated with arthritis [80], psoriasis [27,41], renal transplant rejection [40], chronic airway inflammation [5], inflammatory bowel disease [22], and peritonitis [20,37]. Inflammation is primarily mediated by transcription factors belonging to the nuclear factor-kappa B (NF-κB) family that contains five subunits: p50 (NF-κB1), p65 (Rela), p52 (NF-κB2), RelB, and cRel [23]. All five NF-κB factors contain the Rel homology domain that mediates binding to the prototypic κB element typically present in the promoters of inflammation-responsive genes [73]. The most abundant NF-κB transcription factors are dimers containing p50/p50, p50/p65, or p65/p65 subunits [29,30] that regulate expression of more than 400 proteins implicated in inflammation [1], immunity [23], tumorigenesis [15], angiogenesis [81], and lymphangiogenesis [5,50].

Out of the two most abundant subunits of NF-κB family, p50 and p65, the former appears to play a more prominent role in inducing lymphangiogenesis and the normal

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development of the lymphatic system. This is primarily based on evidence demonstrating constitutive expression of p50 and its precursor protein p105 [64] in the murine lymphatic system and sharp elevation of its expression in response to inflammatory stimuli, tumor necrosis factor-alpha (TNF-α) or LPS [5,37]. Both treatments have been shown to induce lymphangiogenesis [6,37], suggesting that NF-κB p50 might be required for this process. Consistent with this hypothesis, we recently showed in cultured LECs that p50 has a 10-fold higher transactivation potential than p65, and co-transfection of p50 and the VEGFR-3 promoter results in up to 100-fold activation of this promoter [20]. We also showed that p50 was one of the early factors up-regulated during inflammatory lymphangiogenesis in which its increase preceded induction of both VEGFR-3 and the formation of new lymphatic vessels [20].

Another protein coincidently up-regulated with p50 in the thioglycollate (TG) inflammatory model was the homeobox transcription factor, Prox1. This protein defines the lymphatic fate in early venous endothelial cells thus playing a pivotal role in development of embryonic lymphatic system [76,77]. The essential role of Prox1 in embryonic lymphangiogenesis suggests that it might play a similar pro-lymphangiogenic role in postnatal events. Indeed, we showed that the level of Prox1 expression rapidly and sharply increased in the earliest timepoints of inflammation preceding by several days both increased lymphatic expression of VEGFR-3 and genesis of new lymphatic vessels [20]. Moreover, we showed that Prox1 directly binds and activates the VEGFR-3 promoter suggesting that its early increase during inflammation is necessary for elevating VEGFR-3 expression [20]. This finding is in accord with multiple prior studies that showed positive correlation between increased Prox1 and VEGFR-3 expression levels in LEC and blood vascular endothelial cells treated with inflammatory stimuli [20,32,48,56,59].

Taken together the evidence for the regulatory role of Prox1 in VEGFR-3 expression and our novel findings associating NF-κB p50, Prox1 and VEGFR-3, we hypothesized that the absence of NF-κB p50 might both suppress Prox1 and VEGFR-3 expression and attenuate formation of lymphatic vessels. We further hypothesize that if NF-κB p50 plays a role in the initial formation of lymphatic vessels, this might be reflected by reduced lymphatic vessel density (LVD) in normal organs of adult mice with genetically ablated NF-κB p50 (p50 knockout [KO]). To test this hypothesis, we compared LVD in various organs of p50 KO mice with corresponding wild-type (WT) littermates (p50 WT). Unlike embryonically lethal deletion of NF-κB p65 [8], p50 KO mice survive to adulthood but with multifocal defects in response to immune challenge [67]. Neither the blood nor lymphatic vascular phenotype of NF-κB p50 KO mice has been previously investigated. Therefore, the goal of this study was to compare LVD in different organs of p50 KO and WT mice and to correlate LVD with the expression levels of major pro-lymphangiogenic proteins, VEGFR-3 and Prox1. Comparison of LVD between WT and p50 KO mice revealed significantly reduced number of lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1^{+}) vessels in the liver, lung, and in the equivalent of human breast tissue, the mammary fat pad (MFP). In the lung and the liver, this correlated with decreased levels of VEGFR-3 and Prox1 expression measured by qRT-PCR and immunofluorescence. These novel findings provide genetic evidence for the organ-specific role of NF-κB p50 in regulation of VEGFR-3 and Prox1 expression, and optimal LVD in several major normal organs of adult mice.

MATERIALS AND METHODS

Antibodies

Goat anti-mVEGFR-3 and anti-Prox1 antibodies were purchased from R&D Systems (Minneapolis, MN, USA). Rabbit anti-mLYVE-1 and anti-Prox1 antibodies were purchased from AngioBio (Del Mar, CA, USA). Secondary donkey anti-rabbit, anti-goat, and anti-rat antibodies conjugated with DyLight 488 or DyLight 549 were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA).

Animals

Female B6129PF2/J (F2) (p50$^{+/+}$) and B6;129P2-Nfkb 1 < tm 1 Bal> (p50$^{-/-}$) mice four to six weeks of age were obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and treated in accordance with institutional guidelines set by the Animal Care and Use Committee at Southern Illinois University School of Medicine. Mice were anesthetized with a mixture of ketamine (Fort Dodge Animal Health, Fort Dodge, IA, USA), xylazine (Phoenix Scientific Inc., St. Joseph, MO, USA) and sterile water. Prior to tissue harvesting, fully anesthetized mice were perfused with 5 mM $CaCl_2$ solution. Harvested tissues were snap-frozen in liquid nitrogen and then fixed in Shandon Cryomatrix (Thermo Scientific, Waltham, MA, USA) for cryostat sectioning.

Immunofluorescent Staining

Frozen sections were fixed with acetone for ten minutes, washed in PBST (pH 7.4, 0.1% Tween-20) for ten minutes and incubated for two hours at 37°C with primary antibodies (diluted 1:100 in PBST containing 5 μg/mL BSA) against VEGFR-3, LYVE-1, Prox1, or MECA-32, followed by appropriate DyLight 488- or 549-conjugated secondary antibodies (diluted 1:100 in PBST containing 5 μg/mL BSA) for one hour at 37°C. Slides were mounted in Vectashield medium containing 4,6′-diamidino-2-phenylindole

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nuclear stain (Vector Labs, Orton Southgate, UK). Images were acquired on an Olympus BX41 upright microscope equipped with a DP70 digital camera and DP Controller software (Olympus, Center Valley, PA, USA).

Quantification of Vessel Density

Frozen sections of WT and KO organs were acetone-fixed for ten minutes and stained with antibody against the lymphatic marker, LYVE-1 [7], for one hour at 37°C, followed by incubation with DyLight 488-conjugated donkey anti-rabbit secondary antibodies for one hour at 37°C. To quantify LYVE-1 positive vessel density, 3–4 representative images per organ were acquired at 100×, 200×, or 400× magnifications for lungs, MFP, and liver, respectively, and the total number of positive vessels was enumerated and normalized per area of the field (mm^2). LVD is presented as the average number of LYVE-1 positive vessels per area of the field ± SEM (n = 3–5 mice per group).

Measurement of Mean Vascular Area

The mean vascular area of LYVE-1 positive staining per field was calculated as described previously [20], with slight modifications. Briefly, frozen sections were stained with rabbit anti-mLYVE-1 or goat anti-mVEGFR-3 primary antibodies and DyLight 549-conjugated donkey anti-rabbit or donkey anti-goat secondary antibodies, as described above. Fluorescent images were acquired at a constant exposure time at 200× and 400× magnifications for MFP and liver sections, respectively. Images were acquired on an Olympus BX41 upright microscope equipped with a DP70 digital camera and DP Controller software (Olympus). Colored images were sequentially converted to 8-bit grayscale and then to a binary image using Image J software (http://rsbweb.nih.gov/ij/). The total area of LYVE-1 positive staining was then calculated using the analyze particles function of Image J that was set to measure the area of positive staining greater then 10 pixels in size to exclude any background staining. Mean vascular areas were calculated from three images per section ± SEM (n = 3–5 mice per group).

Measurement of Mean Fluorescent Intensity

The mean fluorescent intensity (MFI) of VEGFR-3 and Prox1 positive staining was calculated as described previously [47], with slight modifications. Briefly, frozen sections were stained with goat anti-mVEGFR-3 or goat anti-Prox1 antibodies, followed by incubation with DyLight 549-conjugated donkey anti-goat secondary antibodies as described above. Fluorescent images were acquired at a constant exposure time at 400× magnification on an Olympus BX41 upright microscope equipped with a DP70 digital camera and DP Controller software (Olympus). To exclude background staining, sections stained with secondary antibodies only were used to set the exposure time to below detectable level of background fluorescence. Digital RGB images acquired at a constant exposure time were converted to 8-bit grayscale. The fluorescent intensity for each pixel was calculated using the histogram function of Image J that was set up in the linear intensity range of 0–255 arbitrary units. Staining with secondary antibodies alone resulted in background fluorescence less than 10 units on this scale. MFI was calculated as the sum of the number of pixels above background multiplied by the intensity level in the range of 10–255 and divided by the total pixel number with intensity above 10 units. MFI values were obtained from three images per section (n = 3–5 mice per group) and presented as MFI units ± SEM. To compare the global reduction in fluorescent intensities in p50 KO and WT mice, representative RGB images were converted to 8-bit grayscale and visualized using the 3D interactive surface plot function of Image J.

Quantitative RT-PCR Analysis

Four micrograms of total RNA extracted by Tri-reagent was reverse transcribed using a RevertAid First Strand cDNA synthesis kit, according to the manufacturer's protocol (Fermentas, Burlington, Ontario, Canada). Primers for qRT-PCR were designed against mouse and human CDS of angiogenic and lymphangiogenic proteins found in the NCBI database. Specific primer sequences were chosen using the Harvard primer database website (http://pga.mgh.harvard.edu/primerbank/index.html). All primers were purchased as annealed oligos from Integrated DNA Technologies (Coralville, IA, USA) and sequences of primers used in this study are listed in Table 1. Quantitative RT-PCR was performed using Brilliant II SYBR Green Master Mix (Stratagene, La Jolla, CA, USA) and an ABI 7500 Real-Time PCR machine (Applied BioSystems, Foster City, CA, USA) according to the manufacturer's protocol. A typical qRT-PCR reaction consisted of an initial denaturation step at 95°C for five minutes followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing, extension, and reading at 60°C for one minute. A final melting curve for each primer was calculated by heating from 60 to 90°C. Data were normalized to β-actin and relative mRNA expression was determined using the $\Delta\Delta$Ct method as described previously [66].

Inflammatory Cytokines and Receptors qRT-PCR Array

Two microgram of combined total MFP RNA from p50 KO and WT mice (n = 4 mice per group) was synthesized using a RevertAid First Strand cDNA synthesis kit, according to the manufacturer's protocol (Fermentas). Inflammatory gene expression was examined using a mouse inflammatory cytokines and receptors RT^2 Profiler PCR Array, according to the manufacturer's protocol (PAMM-011, SABiosciences, Fredrick, MD, USA). Target gene expression was normalized

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Table 1. Sequences of primers used for qRT-PCR

qRT-PCR primers	Primer sequences	Product size (bp)
VEGFR-3		
Sense	5′-CTGGCAAATGGTTACTCCATGA-3′	182
Antisense	5′-ACAACCCGTGTGTCTTCACTG-3′	
Proxl		
Sense	5′-TACCAGGTCTACGACAGCACCG-3′	65
Antisense	5′-GTCTTCAGACAGGTCGCCATC-3′	
LYVE-1		
Sense	5′-CAGCACACTAGCCTGGTGTTA-3′	112
Antisense	5′-CGCCCATGATTCTGCATGTAGA-3′	
p-actin		
Sense	5′-GGCTGTATTCCCCTCCATCG-3′	153
Antisense	5′-CCAGTTGGTAACAATGCCATG T-3′	
p65 (Rela)		
Sense	5′-GCTACAC**G**GGACCAGGAACAG-3′*	75
Antisense	5′-AGTTCATGTGGATGAGGCCG-3′	
p52 (NF-κB2)		
Sense	5′-GGCCGGAAGACCTATCCTACT-3′	157
Antisense	5′-CTACAGACACAGCGCACACT-3′	
c-Rel		
Sense	5′-TTGAAGACTGCGACCTCAATG-3′	124
Antisense	5′-GGGGCACGGTTATCATAAATTGG-3′	
Relb		
Sense	5′-CCGTACCTGGTCATCACAGAG-3′	157
Antisense	5′-CAGTCTCGAAGCTCGATGGC-3′	

*Primer detects both rat and mouse p65 with a one base pair mismatch with the mouse sequence denoted in underlined bold.
VEGFR-3, vascular endothelial growth factor receptor-3; LYVE-1, lymphatic vessel endothelial hyaluronan receptor-1.

to β-actin. Relative changes in mRNA expression of p50 KO MFP compared with WT was determined using the $\Delta\Delta$Ct method as described previously [66]. Data are presented as the β-actin normalized relative expression of transcripts in p50 KO MFP (n = 4 mice) compared with WT (n = 4 mice).

Statistical Analysis

Statistical analysis was performed using SAS software (SAS Institute, Inc., Cary, NC, USA). All results are expressed as the mean ± SEM and statistical differences were assessed by unpaired Student's t-test. Statistical significance was defined as $p < 0.05$.

RESULTS

LYVE-1$^+$ Vessel Density is Decreased in Lung, Liver and MFP of p50 KO Mice

Nuclear factor-kappa B dependent induction of inflammatory lymphangiogenesis has been shown in several animal models [5,26,38,41], including evidence obtained in our lab derived from a TG-induced peritonitis mouse model [20]. However, the specific role of the two major NF-κB proteins, p65 and p50, in normal LVD has not been yet examined. The role of p65 is exceedingly difficult to examine postnatally due to embryonic lethality of this genotype [68]. In contrast, p50 KO mice survive to adulthood [24] and present a useful *in vivo* model to clarify the impact of NF-κB p50 on density of lymphatic vessels that are required for the various functions of normal organs.

We previously showed that p50 is a direct transcriptional activator of the VEGFR-3 promoter in cultured LEC and that phosphorylation of p50 precedes both up-regulation of VEGFR-3 and the formation of new lymphatic vessels *in vivo* [20]. Based on these findings, we hypothesized that the absence of p50 may diminish the optimal density of lymphatic vessels in normal organs. To test this hypothesis, we determined LVD in six major normal organs (lung, liver, MFP, kidney, heart, and ovary) of adult female p50 KO and WT mice by enumerating LYVE-1$^+$ vessels. As previously reported [51], in addition to lymphatic vasculature, LYVE-1 was also detected in liver sinusoidal endothelium and thus both vascular types were enumerated. LYVE-1$^+$ lymphatic and sinusoidal vessel density was significantly decreased in three out of six organs of p50 KO mice as compared with WT. Table 2 shows the significant differences detected in: the lung (WT, 966 ± 90 vs. KO, 585 ± 55, $p < 0.001$); the liver (WT, 1307 ± 120 vs. KO, 1133 ± 83, $p = 0.05$); and the MFP (WT, 1917 ± 167 vs. KO, 1569 ± 144, $p < 0.001$). In contrast, kidney, heart, and

Table 2. Lymphatic vessel density (LVD) in normal organs of p50 KO and WT mice

Organ	LVD (per mm)† WT	KO	Percent decrease	p-value
Lung	966 ± 90	585 ± 55*	39.4%	<0.001
Liver	1307 ± 120	1133 ± 83	13.4%	0.05
Mammary fat pad	1917 ± 167	1569 ± 144	18.2%	<0.001
Kidney	11 ± 3	11 ± 2‡	0.0%	n.s.
Heart	125 ± 9	126 ± 10	0.0%	n.s.
Ovary	1049 ± 104	938 ± 184	10.6%	n.s.

†LVD was calculated from frozen organ sections stained with anti-lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1) antibodies. Note that both LYVE-1$^+$ lymphatic and sinusoidal endothelium of the liver were enumerated to calculate hepatic LYVE-1$^+$ vessel density. Three independent images were acquired per animal (n = 3–5 mice per group). Data are presented as mean LVD ± SEM.
‡LVD was normalized to the tissue circumference.
n.s., non-significant changes; WT, wild type; KO, knockout.

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ovary of p50 KO mice showed no significant changes compared with WT (Table 2). This suggested that NF-κB p50 might be important for achieving optimal LVD in the lung, liver, and MFP. However, p50 appears not to play a significant role in generating or maintaining LVD in kidney, heart and ovary.

Decreased LVD Correlates with Suppressed VEGFR-3 and Prox1 Expression in the Lungs of p50 KO Mice

The most conspicuous decrease in LVD in p50 KO mice was in the lung tissues (~40%, Table 2). Because VEGFR-3 and Prox1 are central mediators of lymphangiogenesis [74,77] and their expression has been shown to be regulated by NF-κB p50 [20], we hypothesized that decreased pulmonary LVD might be mediated by deficient expression of VEGFR-3 or Prox1. To test this hypothesis, we compared mRNA levels of LYVE-1 with those of VEGFR-3 and Prox1. The results showed that expression levels of all three lymphatic markers (i.e., LYVE-1, VEGFR-3, and Prox1) were significantly reduced in the lungs of p50 KO mice compared with WT (Table 2). LYVE-1 transcripts were decreased by 32 ± 4% ($p = 0.03$), whereas VEGFR-3 and Prox1 were reduced by 25 ± 10% ($p = 0.17$) and 44 ± 4% ($p = 0.04$), respectively (Table 3). This finding suggests that NF-κB p50 regulates VEGFR-3 and Prox1 expression in lung lymphatic vessels that subsequently might result in reduced LVD in the lungs of p50 KO mice.

To determine whether the levels of VEGFR-3 and Prox1 proteins normalized per LYVE-1$^+$ lymphatic vessel area are also reduced (i.e., relative expression per lymphatic vessel), we calculated the relative MFI in individual lymphatic vessels (described in the Materials and Methods). The MFI values did not differ significantly between WT and KO suggesting that the observed reduction in VEGFR-3 and Prox1 expression levels (Table 3) is due to decreased density of positive vessels rather than to altered protein expression level in individual vessels. To clarify this point, we enumerated VEGFR-3$^+$ and Prox1$^+$ lymphatic vessels and normalized these values per tissue area. This analysis showed a significantly decreased density of VEGFR-3$^+$ and Prox1$^+$ lymphatic vessels by 30% ($p = 0.03$) and 20% ($p = 0.04$), respectively (Figure 1B,C). Moreover, when Prox1$^+$ nuclei were enumerated and normalized per LYVE-1$^+$ lymphatic vessel area, the decrease in Prox1$^+$ nuclei in the lymphatic vasculature of p50 KO mice reached 40% ($p = 0.01$) compared with p50 WT mice (Figure 1D). Collectively, these findings demonstrate that the absence of NF-κB p50 results in down-regulation of the expression of the key lymphangiogenic proteins, VEGFR-3 and Prox1, in adult pulmonary lymphatic vessels.

Decreased Density of LYVE-1$^+$ Vessels Correlates with Reduced Expression of VEGFR-3 in the Liver of p50 KO Mice

LYVE-1$^+$ vessel density in the liver of p50 KO mice was also significantly reduced by 13.4% ($p = 0.05$) as compared with WT mice (Table 2). The reduction in LYVE-1$^+$ vessel density corresponded to a 44% decrease in LYVE-1 transcripts ($p = 0.02$) determined by qRT-PCR (data not shown). In line with decreased LYVE-1 transcripts, VEGFR-3 mRNA was also reduced by 29 ± 6% ($p = 0.004$, Table 3). The reduction in VEGFR-3 mRNA levels also corresponded to decreased expression of VEGFR-3 protein as determined by MFI analysis of slides double-stained with anti-VEGFR-3 and anti-LYVE-1 antibodies (Figure 2).

Table 3. Relative changes in Prox1 and VEGFR-3 expression in normal organs of p50 knockout (KO) *versus* wild-type (WT) mice

Organ	Prox1				VEGFR-3			
	qRT-PCR‡	*p*-value	MFI†	*p*-value	qRT-PCR‡	*p*-value	MFI†	*p*-value
Lung	56 ± 4%	0.04*	104 ± 5%	n.s.	75 ± 10%	n.s.	99 ± 19%	n.s.
Liver	70 ± 5%	0.03	53 ± 3%	<0.001	71 ± 6%	0.004	49 ± 12%	0.002
Mammary fat pad	243 ± 45%	0.02	123 ± 15	0.05	180 ± 29%	0.05	134 ± 12%	0.001
Kidney	90 ± 13%	n.s.	100 ± 9%	n.s.	85 ± 6%	n.s.	100 ± 8%	n.s.
Heart	82 ± 15%	n.s.	98 ± 1%	n.s.	96 ± 6%	n.s.	103 ± 6%	n.s.
Ovary	103 ± 27%	n.s.	85 ± 5%	0.004	126 ± 42%	n.s.	102 ± 9%	n.s.
Brain	49 ± 12%	0.01	75 ± 3%	0.01	109 ± 8%	n.s.	–	–

**p*-value was determined by Student's unpaired *t*-test.
†Mean fluorescent intensity (MFI) presented as the percent expression in p50 KO compared with WT control mice (n = 3–5 mice per group).
‡Transcript expression analyzed by qRT-PCR and presented as the percent expression in p50 KO compared with WT control mice (n = 3–5 mice per group).
VEGFR-3, vascular endothelial growth factor receptor-3; n.s., non-significant changes.

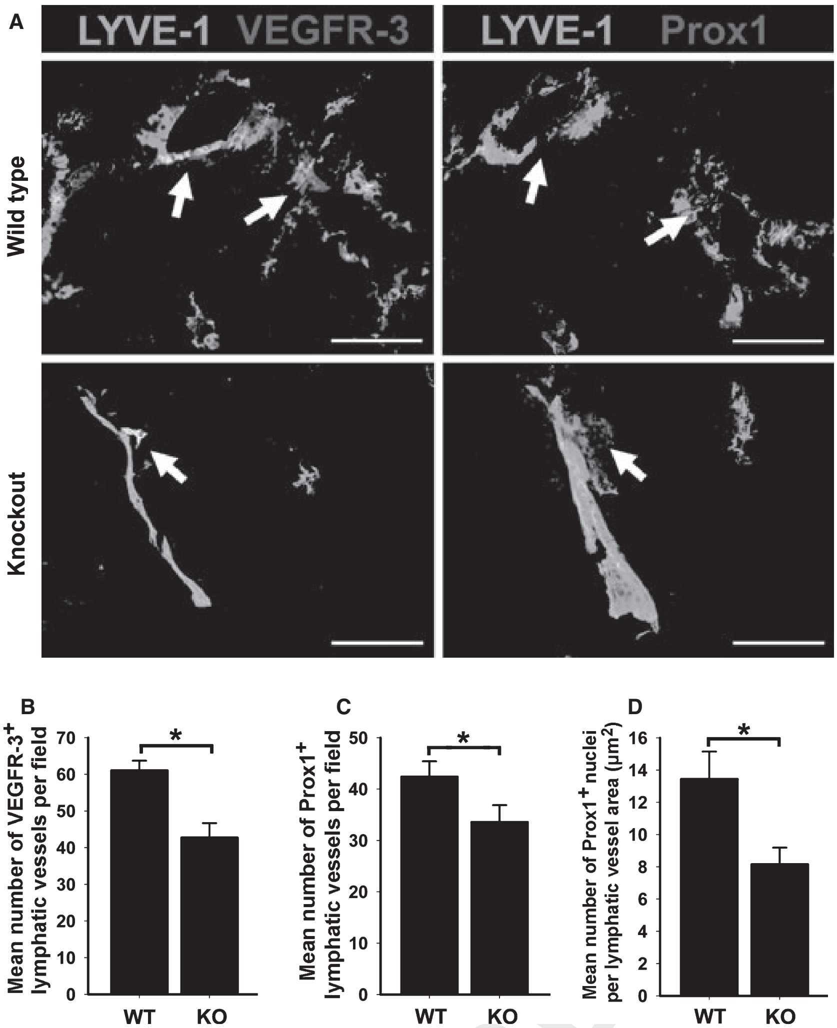

Figure 1. Decreased lymphatic vessel density and reduced expression of Prox1 and VEGFR-3 in the lungs of p50 KO mice compared with WT. (**A**) Double immunofluorescent staining with anti-LYVE-1 and anti-VEGFR-3 or anti-Prox1 antibodies in serial sections of p50 KO and WT lungs, showing reduced lymphatic vessel density. Arrows indicate overlapping expression of VEGFR-3 and Prox1 on LYVE-1^{+} lymphatic vessels on serial sections of p50 KO and WT lungs. Scale bar represents 100 μm. Mean lymphatic vessel density of VEGFR-3 (**B**) and Prox1 (**C**) positive vessels was measured from three images of p50 KO and WT lungs (n = 5 mice per group) acquired at 200× magnification. Data are presented as the mean vascular area ± SEM. The p-values represent *<0.05 and **<0.01 as determined by Student's unpaired t-test. (**D**) The number of Prox1 positive nuclei were enumerated in five images of p50 KO and WT lungs (n = 3–4 mice per group) acquired at 200× magnification. Density of Prox1 positive nuclei was normalized per LYVE-1 positive lymphatic vessel area (μm^2) and is presented as the average number of Prox1 positive nuclei per vessel area ± SEM. The p-value represents *<0.05 as determined by Student's unpaired t-test.

These data indicate that the absence of p50 in the liver causes a coordinated decrease of LYVE-1^{+} vessels and VEGFR-3 expressed on these vessels.

As previously reported, both lymphatic and blood hepatic sinusoidal endothelial cells express LYVE-1 and VEGFR-3 [51,78]. In p50 KO mice, VEGFR-3 protein significantly decreased by 51 ± 12% in both types of vascular cells compared with corresponding cells in WT mice (p = 0.002, Figure 2B and Table 3). Another perturbed vascular parameter in p50 KO livers was marked reduction in the total area of VEGFR-3^{+} and LYVE-1^{+} sinusoidal endothelium (Figure 2A). A similar phenotype has been demonstrated in *Tie2-Cre/IKKβ* mice with targeted disruption of canonical NF-κB p50 and p65 signaling in the endothelial cell compartment [33]. This report and our prior findings in cultured LECs suggested that NF-κB p50-mediated expression of VEGFR-3 might be important for the formation of the LYVE-1-positive endothelium-lined hepatic sinusoids. To confirm this hypothesis, we quantified the mean vascular area of VEGFR-3^{+}/LYVE-1^{+} vessels on images acquired from p50 KO and WT liver sections using Image J software. Compared with WT, the mean vascular area of VEGFR-3^{+} and LYVE-1^{+} vessels in p50 KO livers was significantly decreased by 40% and 55%, respectively (Figure 2C,D). Collectively, these findings suggest that decreased VEGFR-3 expression in p50 KO livers correlates with reduction of both blood and lymphatic vasculature in the livers of p50 KO mice, warranting future studies to interrogate the role of VEGFR-3 signaling in liver development.

Expression of Prox1 is Decreased in both Liver Endothelial Cells and Hepatocytes of p50 KO Mice

We previously reported that lymphatic expression of Prox1 is drastically increased in an inflammatory setting, presumably due to activation of the NF-κB pathway in the lymphatic endothelium [20]. However, the mechanisms regulating Prox1 expression under normal physiological conditions are presently unknown. Based on our prior findings [20], we postulated that NF-κB p50 might be

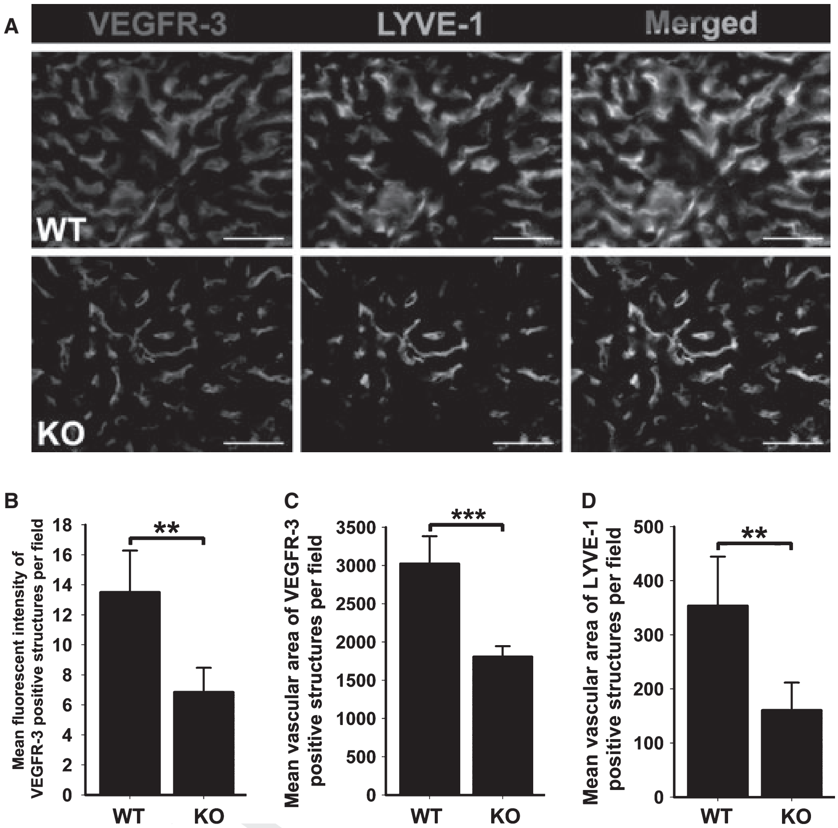

Figure 2. Deletion of NF-κB p50 KO results in decreased expression of VEGFR-3 and LYVE-1 on liver endothelial cells compared with WT. (**A**) Livers of p50 KO and WT mice were double immunostained with anti-VEGFR-3 and anti-LYVE-1 antibodies, showing decreased VEGFR-3 expression in endothelial cells of p50 KO livers compared with WT. Scale bars represent 50 μm. (**B**) The mean fluorescent intensity of VEGFR-3 staining was analyzed in three random fields of p50 KO and WT livers ($n = 5$ mice per group) at 400× magnification. Data are presented as the mean fluorescent intensity ± SEM. The p-value represents **<0.01 as determined by Student's unpaired t-test. The mean vascular area of VEGFR-3 (**C**) and LYVE-1 (**D**) positive staining was calculated from three independent images of p50 KO and WT livers ($n = 5$ mice per group). Data are presented as the mean vascular area normalized per total area ± SEM. The p-values represent **<0.01 and ***<0.001 as determined by Student's unpaired t-test.

required for regulation of Prox1 expression in normal adult organs. The liver is an interesting organ to test this hypothesis because in this tissue Prox1 is expressed in both LECs and hepatocytes [52]. We, therefore, analyzed livers from p50 KO and WT mice for Prox1 mRNA and protein by qRT-PCR and immunofluorescent staining, respectively (Figure 3). As compared with WT mice, expression of both Prox1 mRNA and protein were reduced by ~30% (Figure 3A,B and Table 3) with differences being statistically significant ($p = 0.03$). Reduction in Prox1 protein was observed in both hepatocytes and LYVE-1$^+$ endothelial cells (Figure 3B). Protein levels of Prox1 were also analyzed through comparison of MFI from Prox1-immunofluorescent staining of p50 KO and WT liver sections. This analysis also showed a highly significant ($p < 0.001$) decrease of 47 ± 3% in p50 KO liver cells as compared with WT counterparts (Figure 3C,D and Table 3). These findings indicate that in the context of liver tissue, NF-κB p50 has a significant impact on Prox1 expression in both endothelial and non-endothelial cell types.

Expression of Prox1 is Decreased in the Brain, but not in the Heart, of p50 KO Mice

In addition to hepatocytes, Prox1 has also been detected in several other non-LEC types including cardiomyocytes [62] and neurons [43]. We, therefore, sought to determine whether the absence of p50 in brains and hearts of p50 KO mice causes similar decrease in Prox1 expression as observed in the liver. To answer this question, Prox1 expression was analyzed on mRNA and protein levels using qRT-PCR and MFI, respectively. In the brain of p50 KO mice, transcripts and protein levels of Prox1 were reduced by 51 ± 12% and 25 ± 3%, respectively, compared with WT (Figure 4A–C and Table 3), with both values being statistically significant ($p = 0.01$, Table 3). In contrast, no change in Prox1 expression was observed in the heart of p50 KO (data not shown), suggesting that the regulatory effect of p50 NF-κB on Prox1 expression might be tissue-specific, being prominent in some organs but dispensable in others. This is consistent with similar tissue-dependent variable effects of deletion of p50 NF-κB that have been previously reported [67].

The MFPs of p50 KO Mice Exhibit Decreased LYVE-1$^+$ LVD but Aberrantly Increased VEGFR-3 and Prox1 Expression on Lymphatic Vasculature

The mouse MFP is a tissue with very high LVD and expression of lymphatic-specific proteins. To analyze LVD in the MFP of KO and WT p50 mice, we compared the numbers of anti-LYVE-1 antibody stained structures in

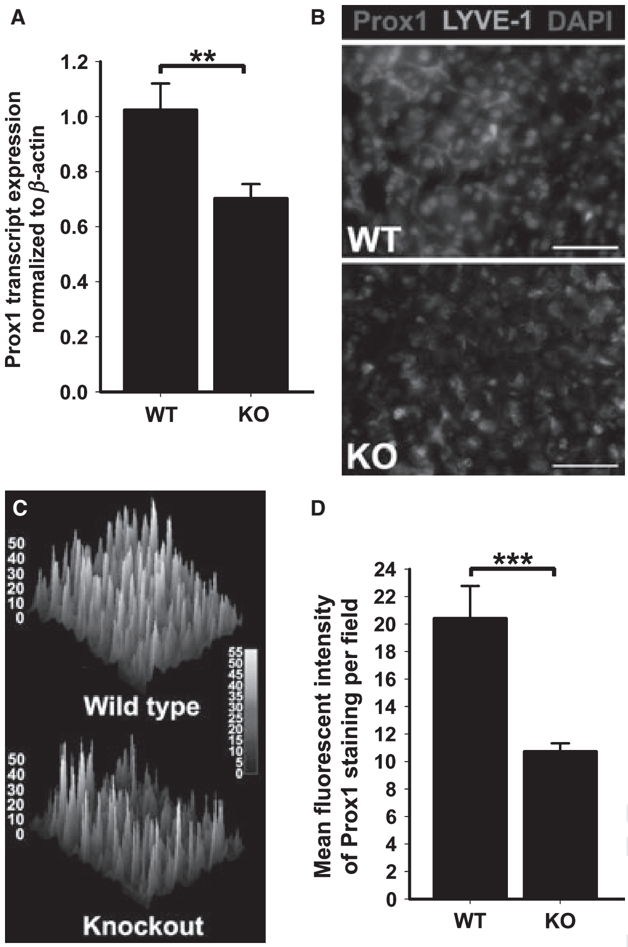

Figure 3. Expression of Prox1 in the mouse liver is significantly decreased by deletion of NF-κB p50 KO as compared with WT. (**A**) Quantitative RT-PCR analysis of Prox1 transcript expression in total RNA extracted from p50 KO and WT livers (n = 5 mice per group). Relative expression was normalized to β-actin. Data are presented as mean transcript expression ± SEM. p-values are indicated as *<0.01 as determined by Student's unpaired t-test. (**B**) Liver sections were double immunostained with anti-Prox1 and anti-LYVE-1 antibodies. Scale bars represent 50 μm. (**C**) A representative surface plot of the fluorescent intensity of Prox1 staining in p50 KO and WT liver sections. Note in panels (**B**) and (**C**) a dramatic reduction in Prox1 expression in both liver sinusoidal endothelial cells and hepatocytes. (**D**) The mean fluorescent intensity of Prox1 staining was analyzed in three random fields of p50 KO and WT livers (n = 5 mice per group) acquired at 400× magnification. Data are presented as the mean fluorescent intensity ± SEM. The p-value represents ***<0.001 as determined by Student's unpaired t-test.

corresponding tissues (Figure 5A). This analysis revealed a ~20% decrease in density of LYVE-1$^+$ lymphatic vessels ($p < 0.001$, Table 2 and Figure 5B) and ~60% decrease in mean vascular area of LYVE-1$^+$ staining per section area in p50 KO mice ($p < 0.001$, Figure 5C). This finding was corroborated by qRT-PCR analysis that detected a corresponding 66 ± 9% decrease in LYVE-1 transcripts (p = 0.01, Figure 5D). Surprisingly, however, the number of VEGFR-3$^+$ and Prox1$^+$ lymphatic vessels was slightly higher in p50 KO mice compared with WT, although the differences did not reach statistical significance (data not shown). Additionally, the MFI of VEGFR-3 and Prox1 in lymphatic vessels was elevated by 134 ± 12% (p = 0.001) and 123 ± 15% (p = 0.05), respectively, in p50 KO mice compared with WT (Table 3). This was corroborated by statistically significant 1.8-fold and 2.4-fold increases in VEGFR-3 and Prox1 transcripts, respectively, as detected by qRT-PCR (Table 3). These findings indicated that although the density of LYVE-1$^+$ lymphatic vessels was reduced in the MFPs of p50 KO mice, the expression levels of VEGFR-3 and Prox1 per lymphatic vessel were significantly increased. These findings suggested that in the MFP context, p50 ablation reduced LVD despite overexpression of some lymphatic markers through possibly compensatory regulatory mechanisms.

Elevated VEGFR-3 and Prox1 Expression on Lymphatic Vasculature of the MFPs of p50 KO Mice is Associated with Altered Profile of Inflammatory Mediators

To explain the paradoxical increase of VEGFR-3 and Prox1 in MFP lymphatic vessels, we hypothesized that the absence of p50 in this tissue is compensated by other members of the NF-κB family whose pro-inflammatory signaling up-regulates VEGFR-3 and Prox1 expression but is insufficient for generation of new lymphatic vessels. This is plausible because NF-κB subunits p65 and p52 have been previously reported to compensate for the lack of p50 [79]. To determine whether these subunits could be differentially expressed in tissues of p50 KO and WT mice, we analyzed p65 and p52 expression levels by qRT-PCR. This analysis revealed a nearly perfect correlation between changes in expression levels of VEGFR-3 and NF-κB p65 in each organ examined (Table 4). For example, both VEGFR-3 and p65 were reduced in lung and liver, elevated in MFP, and close to level of WT mice in kidney, heart, and brain. Similarly, the pattern of NF-κB p52 expression was highly correlative with Prox1 profile, with both genes being reduced in lung, liver and brain, elevated in MFP, and roughly unchanged in kidney and heart (Table 4). These data suggested that in the absence of p50 VEGFR-3 and Prox1 expression might be regulated by p65 and p52 NF-κB subunits overexpressed in the MFP of p50 KO mice.

Positive correlation between expression levels of VEGFR-3 and Prox1 and p65/p52 NF-κB subunits suggested that the MFP tissue compensates for the lack of p50 by up-regulating both canonical (p65) and non-canonical (p52) NF-κB pathways. This finding suggested that overexpression of p50-alternative transcription factors might create a pro-inflammatory rather than an anti-inflammatory

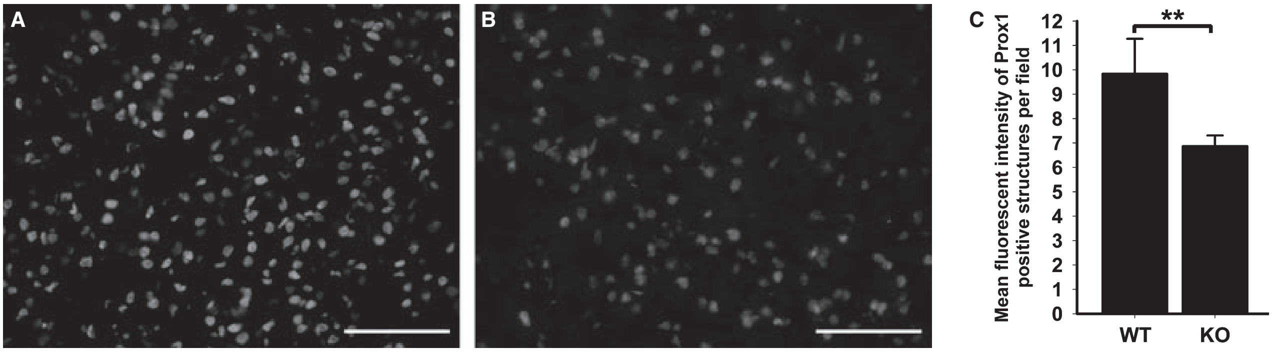

Figure 4. Cell-type specific downregulation of Prox1 in neurons of p50 KO brains compared with WT. Frozen sections of p50 WT (**A**) and KO (**B**) brains were immunostained with anti-Prox1 antibodies and counterstained with DAPI. Note the dramatic reduction in the fluorescent intensity of Prox1 immunostaining in p50 KO compared with WT. (**C**) Analysis of the MFI of Prox1 staining in p50 KO and WT neurons (n = 3–4 mice per group). Data are presented as MFI of Prox1 staining ± SEM. The p-value represents **<0.01 as determined by Student's unpaired t-test.

environment. To test this hypothesis, we compared the expression levels of 84 NF-κB-dependent genes in the MFP of p50 KO and WT mice by using an inflammation gene-targeted PCR array. We found that a large portion of the tested genes (35 of 84, or 41.6% of total) expressed in the MFP of p50 KO mice were elevated by at least 1.5-fold compared with the MFP gene profile in WT mice (Table 5). A smaller fraction (28.5%) was down-regulated in p50 KO MFPs by at least 1.5-fold, while the level of remaining genes was unchanged. This finding suggested that VEGFR-3 and Prox1 are upregulated in MFPs of NF-κB p50 KO mice due to aberrant inflammation induced by compensatory over-expression of two factors of the NF-κB family, p65 and p52. However, the pathways induced by these two transcription factors were insufficient for reaching normal LVD mediated by p50 subunit as indicated by the 18% reduction in LVD in MFPs of p50 KO mice as compared with WT (Table 2). This evidence further underscores the essential role of the NF-κB p50 subunit in postnatal formation of lymphatic vessels in normal tissues.

DISCUSSION

Ablation of NF-κB p50 Decreases Lymphatic Vessel Density and Lymphatic VEGFR-3 Expression in some Normal Organs

We previously reported that the two most abundant NF-κB transcription factors, p50 and p65, are elevated prior to inflammatory lymphangiogenesis induced in a mouse peritonitis model *in vivo* [20]. We also showed in cultured LECs that p50, and to a lesser extent p65 protein, bind and activate the human VEGFR-3 promoter and induce its transcription [20]. Additionally, our data demonstrated that p50 but not the p65 subunit of NF-κB synergizes with Prox1, a lymphatic-expressed transcription factor, in up-regulating VEGFR-3 [20]. These data indicated a major role for NF-κB proteins, and in particular, NF-κB p50, in controlling the pro-lymphangiogenic intracellular milieu necessary for creating and maintaining the lymphatic vasculature. Other groups have demonstrated that NF-κB proteins, and particularly p50 subunit, are constitutively expressed in normal adult lymphatic endothelium [64] and contribute to normal function of lymphatic vessels in adults [37].

Based on these prior data and reports, we hypothesized that mice lacking the NF-κB p50 subunit might have lower expression of Prox1 and VEGFR-3 that subsequently would result in reduced LVD in normal organs. Tissue analysis from p50 KO mice confirmed that indeed three out of six organs with lymphatic networks (e.g., lung, liver, and MFP) exhibited decreased LYVE-1^{+} vessel density (Figures 1 and 2 and Table 2). Moreover, reduction of LVD in most affected organs corresponded to significant suppression of VEGFR-3 and Prox1 expression (Tables 2 and 3) suggesting that these genes are constitutively regulated by p50 on a transcriptional level. This is in line with other studies reporting that constitutive activity of NF-κB p50 is essential for maintaining various immune functions under normal conditions [10,11,19,54]. This conclusion is also consistent with our prior findings demonstrating that silencing NF-κB expression significantly reduced levels of Prox1 and VEGFR-3 in untreated cultured LEC [20], therefore suggesting that p50 is important for normal LEC functions. Two independent publications support this conclusion by demonstrating constitutive activity of the promoter of p105 (a p50 precursor) in the LEC of transgenic *κB-lacZ* mice that underwent further up-regulation upon exposure to inflammatory factors [37,64]. Collectively, these data suggest that NF-κB p50 regulates expression of VEGFR-3 and Prox1 in LEC under both normal and inflamed conditions, and that the level of these proteins is important for normal lymphatic vessel development in some organs.

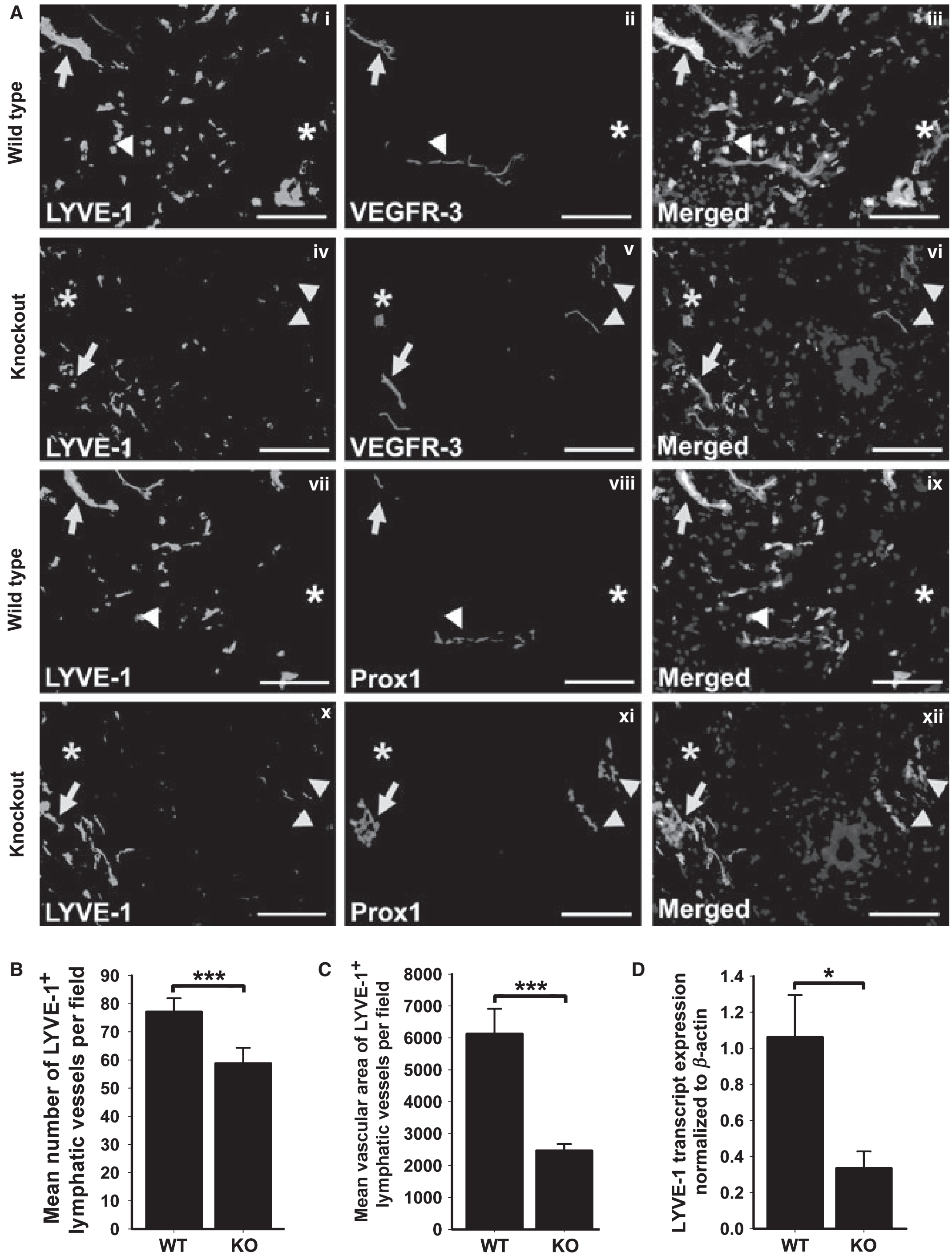

Figure 5. Decreased LYVE-1$^+$ lymphatic vessel density despite elevated expression of VEGFR-3 and Prox1 in the MFP of p50 KO mice compared with WT. (**A**) Serial frozen sections of p50 KO and WT MFP were double immunostained with anti-LYVE-1 and anti-VEGFR-3 (i–vi) or anti-LYVE-1 and anti-Prox1 (vii–xi) antibodies. Scale bar represents 100 μm. Some VEGFR-3$^+$/Prox1$^+$ lymphatic vessels also expressed LYVE-1 (arrows), whereas other VEGFR-3$^+$/Prox1$^+$ lymphatic vessels were LYVE-1 negative (arrowhead). Vascular structures resembling blood vessels also stained positive for VEGFR-3 but negative for lymphatic markers Prox1 and LYVE-1 (asterisk). Note that overall density and expression of VEGFR-3$^+$/Prox1$^+$ was increased in p50 KO (iv–vi and x–xii) compared with WT (i–iii and vii–ix). (**B**) LYVE-1 positive lymphatic vessels were enumerated in six images per p50 KO and WT MFPs (n = 3 mice per group) acquired at 200× magnification. The results are presented as the mean LYVE-1 positive vessel density per 200× field ± SEM. The p-value represents ***<0.001 as determined by Student's unpaired t-test. (**C**) The mean vascular area of LYVE-1 positive vessels was measured from three images of p50 KO and WT MFPs (n = 3 mice per group) acquired at 200× magnification. The p-value represents ***<0.001 as determined by Student's unpaired t-test. (**D**) Quantitative RT-PCR analysis of LYVE-1 was performed using total RNA extracted from p50 KO and WT MFPs (n = 4–5 mice per group). Relative expression was normalized to β-actin. Data are presented as the mean β-actin normalized transcript expression ± SEM. The p-value represents *<0.05 as determined by Student's unpaired t-test.

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Table 4. Relative expression of Prox1 and VEGFR-3 transcripts compared to expression of nuclear factor-kappa B p65 and p52 subunits in p50 knockout *versus* wild-type mice

Tissue†	Prox1	VEGFR-3	p65 (Rela)	*p*	p52 (NF-κB2)	*p*
Lung	56 ± 4%	75 ± 10%	58 ± 5%	<0.001	57 ± 10%	0.05
Liver	70 ± 5%	71 ± 6%	79 ± 2%	0.002*	76 ± 5%	0.05
Mammary fat pad	243 ± 45%	180 ± 29%	140 ± 4%	0.001	158 ± 2%	0.005
Kidney-	90 ± 13%	85 ± 6%	100 ± 6%	n.s.	88 ± 10%o	n.s.
Heart	82 ± 15%	96 ± 6%	90 ± 3%	0.03	69 ± 2%	<0.001
Brain	49 ± 12%	109 ± 8%	97 ± 7%	n.s.	76 ± 5%	0.03

**p*-value was determined by Student's unpaired *t*-test.
†cDNA synthesized from total RNA extracted from each tissue (n = 3–5 mice per group).
VEGFR-3, vascular endothelial growth factor receptor-3; n.s., non-significant changes.

In contrast to the lung, liver, and MFP, several other organs (e.g., kidney, heart, and ovary) of NF-κB p50 KO mice showed no significant changes in LVD or VEGFR-3 and Prox1 expression (Tables 2 and 3). In these unaffected p50 KO organs, expression of NF-κB p65 remained close to or equal to the normal levels in WT mice (Table 5), suggesting that signaling through p65 homodimers or p65/p52 heterodimers might compensate for the lack of p50 in an organ-specific manner (summarized in Figure 6). The maintenance of the inflammatory milieu by p65 in the absence of p50 has been previously shown in inflamed hearts and kidneys of p50 KO mice [57,70]. Inflammatory stimuli that activate NF-κB p65 homodimers (e.g., LPS and TNF-α) are also known to induce lymphangiogenesis [6,37], suggesting that under some circumstances NF-κB p65 homodimers are sufficient to regulate both inflammation-induced lymphangiogenesis and normal lymphatic vessel development. Due to the embryonic lethality of p65 KO mice [8], it is currently not possible to determine whether NF-κB p65 is essential for postnatal lymphangiogenesis under normal conditions. However, lack of differences in LVD in aforementioned organs strongly suggests that p65 and the non-canonical NF-κB subunits might compensate for the absent function of p50 protein.

Tissue-specific regulation of VEGFR-3 and Prox1 expression might also be mediated by differential expression of NF-κB p50 binding partners, such as NF-κB p65, that are required for activating gene transcription, because NF-κB p50 lacks a consensus transactivation domain [9]. Moreover, relative expression of NF-κB p50 and p65 subunits under different conditions and cellular contexts likely modulates the ratio of p50/p65 heterodimers and p50/p50 or p65/p65 homodimers. This may have significant impact on gene regulation because all NF-κB dimers are able to bind to the same κB response element [13,30], albeit with variable affinity [13,30] and functional consequences (i.e., promoter activation or repression) [13,30]. Presence or absence of other transcription factors and regulatory proteins outside the NF-κB family may also modulate NF-κB p50 function. NF-κB p50/p50 homodimers interact with the transcriptional co-activators, such as Bcl-3 [42] or C/EBP proteins [14], that determine whether p50/p50 functions as a transcriptional activator (e.g., Bcl-2 [42] and PDGF-A promoters [2]) or repressor (e.g., TNF-α and interleukin[IL]-8 promoters [72]). We recently have shown that co-expression of Prox1 with NF-κB p50 synergistically activated the VEGFR-3 promoter [20], suggesting that Prox1 might be a lymphatic-specific co-activator of p50. This may be possible through recruitment of the common binding partner and transcriptional co-activator, CBP/p300 [16]. Moreover, synergy between NF-κB p50 and Prox1 and evidence that NF-κB p50 may regulate Prox1 expression (Table 2, and Figures 1 and 3), suggests that Prox1 may affect the function of NF-κB p50 in two ways: by amplifying p50 signaling and by imparting lymphatic specificity to the activated NF-κB pathway.

Ablation of NF-κB p50 in the MFP Creates Abnormal Environment Characterized by Reduced LVD but Elevated VEGFR-3 and Prox1

The lymphatics in the MFP of p50 KO mice were uniquely affected by the absence of NF-κB p50: similarly to lungs and liver, LYVE-1$^+$ vessel density was significantly reduced (Figure 5 and Table 2), however, the expression of VEGFR-3 and Prox1 was paradoxically elevated (Table 3). We drew two conclusions from these observations: (i) up-regulation of Prox1 and VEGFR-3 suggests compensatory overexpression of p50-alternative NF-κB factors; and (ii) normal MFP lymphatic vessel development might require p50-specific signals other than Prox1 and VEGFR-3. Both of these conclusions are supported by data presented here and are plausible based on the findings of previous reports. Tables 4 and 5 show that, in contrast to other organs, the MFP of p50 in KO mice is characterized by significantly increased

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Table 5. Changes in expression of inflammatory mediators in MFPs of p50 KO *versus* WT mice

Gene name	Symbol	Fold change compared with WT	Gene name	Symbol	Fold change compared with WT
Interleukin 17†	Il17b	9.25*	Chemokine (C–C motif) ligand 20	Ccl20	1.16
Complement component 3	C3	6.06	Interleukin 4	Il4	1.16
Chemokine (C–C motif) receptor 10	Ccr10	5.86	Interleukin 16	Il16	1.14
Tumor necrosis factor receptor superfamily, member 1a	Tnfrsfla	4.72	Chemokine (C–C motif) ligand 17	Ccl17	1.12
			Interferon gamma	Ifng	1.10
Macrophage migration inhibitory factor	Mif	3.89	Chemokine (C–C motif) ligand 24	Ccl24	1.10
Interleukin 13 receptor, alpha 1	Il13ra1	3.61	Chemokine (C–C motif) receptor 9	Ccr9	1.09
Small inducible cytokine subfamily E, member 1	Scyel	3.27	Interleukin 1 alpha	Il1a	1.07
			Chemokine (C–C motif) ligand 19	Ccl19	1.06
Interleukin 6 signal transducer	Il6st	3.12	Chemokine (C–C motif) receptor 4	Ccr4	1.06
Interleukin 18	Il18	2.83	Chemokine (C–X–C motif) ligand 5	Cxcl5	1.06
ATP-binding cassette, sub-family F (GCN20), member 1	Abcf1	2.73	Chemokine (C–C motif) ligand 3	Ccl3	1.01
			Interleukin 1 receptor, type II	Il1r2	1.00
Chemokine (C–X–C motif) ligand 15	Cxcl15	2.68	Chemokine (C–C motif) receptor 2	Ccr2	0.93
Chemokine (C–C motif) ligand 11	Ccl11	2.62	Chemokine (C–C motif) ligand 6	Ccl6	0.90
B-cell leukemia/lymphoma 6	Bcl6	2.60	Chemokine (C–C motif) ligand 9	Ccl9	0.88
Chemokine (C–X3–C motif) ligand 1	Cx3cl1	2.60	Interleukin 10 receptor, alpha	Il10ra	0.87
Chemokine (C–C motif) receptor 5	Cxcr5	2.51	Chemokine (C–C motif) ligand 7	Ccl7	0.84
C-reactive protein, pentraxin-related	Crp	2.50	Integrin alpha M	Itgam	0.81
Chemokine (C–X–C motif) ligand 12	Cxcl12	2.28	Chemokine (C–C motif) ligand 2	Ccl2	0.77
Interleukin 15	Il15	2.27	Interleukin 1 beta	Il1b	0.76
Secreted phosphoprotein 1	Spp1	2.17	Chemokine (C–C motif) ligand 4	Ccl4	0.69
Interleukin 1 receptor, type I	Il1r1	1.93	Chemokine (C–C motif) ligand 12	Ccl12	0.66
Interleukin 1 family, member 8	Il1f8	1.85	Chemokine (C motif) receptor 1	Xcr1	0.64
Chemokine (C–C motif) receptor 1	Ccr1	1.80	Integrin beta 2	Itgb2	0.62
Chemokine (C–X–C motif) ligand 9	Cxcl9	1.74	Interleukin 5 receptor, alpha	Il5ra	0.60
Interleukin 11	Il11	1.74	Chemokine (C–C motif) ligand 1	Ccl1	0.59
Transforming growth factor, beta 1	Tgfb1	1.74	Chemokine (C–X–C motif) ligand 1	Cxcl1	0.59
Chemokine (C–X–C motif) ligand 10	Cxcl10	1.71	Interleukin 8 receptor, beta	Il8rb	0.59
Chemokine (C–X–C motif) ligand 11	Cxcl11	1.69	Interleukin 10	Il10	0.48
Interleukin 6 receptor, alpha	Il6ra	1.69	Chemokine (C–X–C motif) receptor 3	Cxcr3	0.45
Toll interacting protein	Tollip	1.69	Interleukin 3	Il3	0.39
Caspase 1	Casp1	1.68	Interleukin 2 receptor, beta chain	Il2rb	0.37
Tumor necrosis factor receptor superfamily, member 1b	Tnfrsf 1b	1.65	Chemokine (C–X–C motif) ligand 13	Cxcl13	0.36
			Chemokine (C–C motif) receptor 8	Ccr8	0.28
Platelet factor 4	Pf4	1.61	Lymphotoxin A	Lta	0.24
Chemokine (C–C motif) ligand 8	Ccl8	1.59	Chemokine (C–C motif) receptor 7	Ccr7	0.24
Interleukin 10 receptor, beta	Il10rb	1.57	Interleukin 2 receptor, gamma chain	Il2rg	0.16
Chemokine (C–C motif) receptor 3	Ccr3	1.51	Chemokine (C–C motif) receptor 6	Ccr6	0.16
Tumor necrosis factor	Tnf	1.49	Chemokine (C–C motif) ligand 5	Ccl5	0.13
Chemokine (C–C motif) ligand 25	Ccl25	1.43	Chemochine (C–X–C motif) receptor 5	Ccr5	0.12
Interleukin 13	Il13	1.38	Lymphotoxin B	Ltb	0.07
Interleukin 20	Il20	1.27	CD40 ligand	Cd40lg	0.05
Interleukin 1 family, member 6	Il1f6	1.26	Chemokine (C–C motif) ligand 22	Ccl22	0.04

*Fold changes were derived from qRT-PCR array analysis of total MFP mRNA from p50 KO and WT mice (n = 4 mice per group).
†Gray shading indicates genes with greater than 0.5-fold change expression in p50 KO compared with WT mammary fat pad.
KO, knockout; WT, wild type; MFP, mammary fat pad.

expression of NF-κB p65 and p52 subunits as well as a greater than 150% increase in expression levels of 35 inflammatory cytokines. These findings are consistent with a previous report demonstrating that p52 NF-κB might compensate for the lack of p50 signaling in TNF-α treated mammary epithelial cells [79]. Additionally, NF-κB dimers that do not include the p50 subunit (e.g., p65/p65 and p65/p52) are known to bind and activate κB response elements [65], suggesting that in the absence of p50 these dimers could independently activate the VEGFR-3 and

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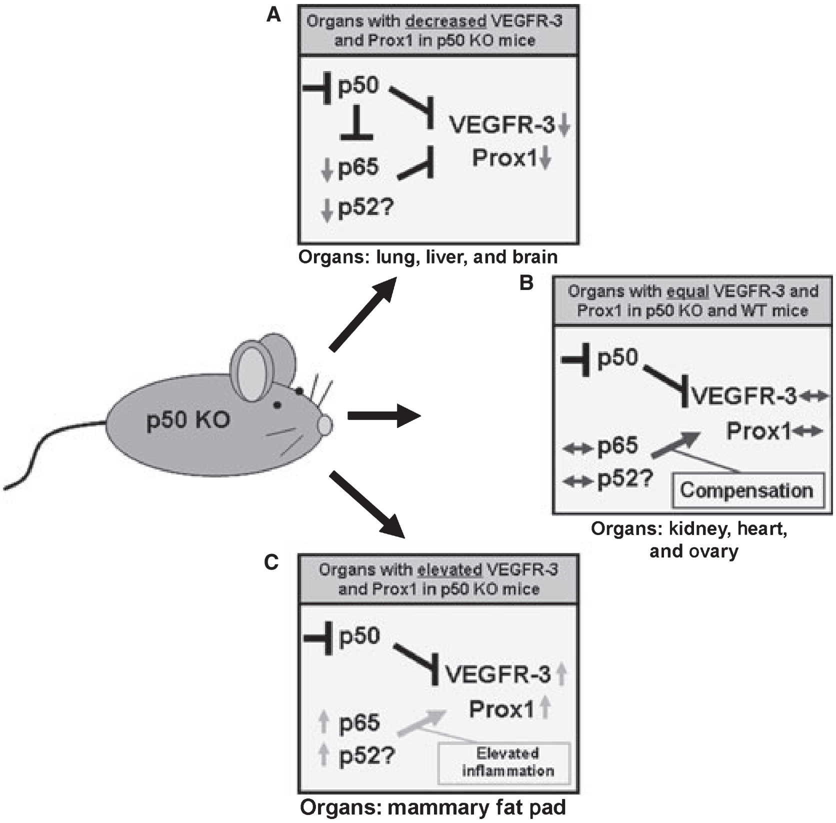

Figure 6. Potential impact of NF-κB p50 deletion on expression of VEGFR-3 and Prox1. (**A**) Expression of VEGFR-3 and Prox1 was significantly downregulated in several organs of p50 KO mice (e.g., lung, liver, and brain), which correlated with a global reduction of the other major NF-κB transcription factors, p65 and p52. This is consistent with previous findings that several NF-κB subunits are variably downregulated in a cell-type specific manner in different tissues of p50 KO mice [67]. This suggests that p50 along with p65 and p52 might be involved in transcriptional regulation of VEGFR-3 and Prox1 expression. (**B**) In comparison, deletion of NF-κB p50 did not affect expression of NF-κB p65 and p52 in the kidney, heart, and ovary of p50 KO mice, corresponding to equal expression of VEGFR-3 and Prox1 in the same p50 KO organs compared to WT. This suggests that in these organs p50 may be dispensable for regulation of expression of VEGFR-3 and Prox1 and other subunits of NF-κB (e.g., p65 and p52). (**C**) In one p50 KO tissue, the mammary fat pad, the expression of NF-κB p65 and p52 was dramatically increased along with elevated expression of 35 inflammatory cytokines (Table 5). This corresponded to significantly increased levels of VEGFR-3 and Prox1 expression, despite the lack of the p50 subunit of NF-κB. **For both panels B and C:** Since NF-κB p65 and p52 have previously been shown to bind and activate NF-κB p50 response elements (i.e., κB sites), this implies that NF-κB p65 and p52 might potentially compensate or overcompensate for the lack of NF-κB p50 signaling in certain organs. Furthermore, NF-κB p65 was previously shown to independently regulate the VEGFR-3 promoter and be required for expression of Prox1 in cultured LECs [20], further suggesting that other NF-κB subunits may regulate expression of VEGFR-3 and Prox1 in addition to p50.

Prox1 promoters. This is also supported by our recent finding that p65 binds and activates the VEGFR-3 promoter [20], albeit with much lower efficiency than the p50 subunit. Collectively, these data indicate that increased expression of p65 and p52 in p50 KO MFP compensates for the lack of p50 by increasing the expression of VEGFR-3 and Prox1 (summarized in Figure 6). However, the increased levels of these pro-lymphangiogenic factors was insufficient to fully compensate for optimal lymphatic vessel development observed in the MFP of WT mice.

The later conclusion implies that normal MFP lymphangiogenesis requires some p50-specific factors in addition to VEGFR-3 and Prox1. Table 5 shows that approximately 15 of NF-κB p50-dependent inflammatory cytokines, including IL-3, lymphotoxin α, and lymphotoxin β, are down-regulated by more than 2-fold in MFPs of p50 KO mice. This is in line with previous findings demonstrating that lymphotoxins α and β as well as IL-3 are direct activators of LEC and lymphangiogenesis *in vivo* [20,21,25,44,49,58]. Taken together, the data presented here suggest that p50-dependent IL-3 and lymphotoxin pathways might be important for maintaining normal MFP lymphatic vessel density.

Regulation of Lymphatic-Specific VEGFR-3 and Prox1 Expression by NF-κB p50 *In Vivo*

Although Prox1 has been shown to regulate VEGFR-3 in cultured endothelial cells [20,32,48,56,59], very few studies

correlated Prox1 expression in relation to VEGFR-3 *in vivo*. We have previously shown that Prox1 binds and activates the VEGFR-3 promoter [20], suggesting that Prox1 is a direct transcriptional regulator of the VEGFR-3 gene. This is consistent with novel findings presented here that demonstrate complete overlap between expression patterns of Prox1 and VEGFR-3 in normal organs of p50 KO as compared with WT mice (Table 3). These data support the notion that one of the functions of Prox1 in adult LEC is to control levels of VEGFR-3 expression that are required for robust lymphangiogenic response.

The other important and currently open question in this context is the identity of factors that control Prox1 expression, particularly those that elevate its expression in LEC and suppress it in most non-lymphatic cell types. We previously reported that NF-κB-dependent inflammation induced lymphatic-specific Prox1 expression, which preceded inflammatory lymphangiogenesis *in vivo* [20]. Additionally, using *in vitro* techniques, we also demonstrated that manipulating NF-κB p50 signaling correlated with Prox1 expression in LEC [20]. Similar results have been obtained in several independent studies showing that Prox1 is increased in endothelial cells treated with IL-3 [25] or another potent activator of the NF-κB pathway, Kaposi Sarcoma Herpes Virus [31]. The notion that Prox1 might be regulated by NF-κB p50 is also supported by the evidence from this study demonstrating decreased expression of Prox1 in the liver, lungs, and brain of p50 KO mice (Table 3). However, other organs were not affected suggesting the existence of additional tissue-specific factors controlling Prox1 expression. This tissue-dependent variability in Prox1 expression and corresponding defects in lymphatic vasculature is similar to findings in *Fiaf*$^{-/-}$ mice that have reduced Prox1 expression and lymphatic vascular defects in the small intestine, but normal level of Prox1 and unaffected lymphatic vasculature in all other organs [4]. The tissue-specific regulation of Prox1 expression may also account for the parallel normal levels of VEGFR-3 expression in some organs tested here, further supporting our previous observations that Prox1 may activate the VEGFR-3 promoter in both p50-dependent and independent manners [20].

NF-κB p50 also Regulates Prox1 in some Non-Lymphatic Cell Types

Although Prox1 is considered a lymphatic-specific marker [36], its expression has also been reported in various non-lymphatic tissues including liver [69], brain [43], heart [62], pancreas [75], eye [18,71], ear [17], and small intestine [60]. In line with these observations, we detected Prox1 in non-LECs in several normal organs of p50 KO and WT mice, including liver hepatocytes, brain neurons, and heart cardiomyocytes. Although no changes in Prox1 expression were detected in cardiomyocytes, this protein was significantly downregulated in both hepatocytes and neurons of p50 KO mice compared with WT (Figures 3 and 4 and Table 3). This indicates that NF-κB p50 regulates Prox1 not only in lymphatic endothelium but also in some non-lymphatic cell types.

Prox1 was reported to play an essential role in liver morphogenesis [69], hepatocyte migration [69], and liver metabolism [12]. No changes, however, were previously noted in the liver function of NF-κB p50 KO mice [39], suggesting that the remaining ~50% of Prox1 protein (Figure 3D) is sufficient for normal hepatogenesis. In contrast, genetic ablation of p50 was reported to impair hippocampal neurogenesis [34] and to increase age-related neural degeneration [45]. The molecular mechanisms underlying these disorders in p50 KO mice are currently unclear. Our discovery that Prox1 expression in non-lymphatic tissues (e.g., brain) is potentially regulated by NF-κB p50 might open new avenues in understanding of the function of Prox1 in adult tissues under normal, inflamed and pathological circumstances. Future validation of NF-κB mediated control of Prox1 expression is warranted because of the significance of Prox1 function in both lymphatic endothelium and other cell types.

CONCLUSIONS

This work was initially inspired by the impact of the NF-κB p50 subunit on transactivation of VEGFR-3 promoter [20] and subsequently evolved to a systematic comparison of VEGFR-3 and Prox1 expression in the lymphatics of normal organs in p50 KO and WT mice. During this analysis, we have made several novel observations such as: (i) original evidence for the role of NF-κB p50 in organ-specific maintenance of LVD and expression of the key pro-lymphangiogenic proteins, VEGFR-3 and Prox1, under normal conditions; (ii) strong associations among VEGFR-3, Prox1 and LVD that are consistent with the notion that NF-κB regulates Prox1 and VEGFR-3 expression *in vivo*; and (iii) novel evidence demonstrating the suppression of Prox1 expression in p50 KO hepatocytes and neurons, suggesting for the first time that NF-κB p50 regulates Prox1 expression in non-lymphatic cell types. Although these data support the idea that NF-κB p50 is involved in regulation of VEGFR-3 and Prox1 expression, transcription of these genes might be additionally controlled by p65 and p52 as well as by other regulatory factors outside of the NF-κB family. Nevertheless, our observations are important for documenting LVD and expression levels of major lymphangiogenic factors in adult normal organs while highlighting the possible roles of the NF-κB p50 subunit in normal physiology of both endothelial and non-endothelial tissues.

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ACKNOWLEDGEMENTS

The authors thank Dr. Linda Toth for providing p50 WT and KO mice used in the initial experiments. We also gratefully thank Heather McKinnon, Kathleen Brancato, and Shelly Reeter for their assistance in tissue sectioning and immunostaining. This work was supported by grants from the National Institute of Health (#1R15CA125682–01 and #1R01CA140732-01A1), Illinois William E. McElroy Foundation, and SIUSOM Excellence in Medicine awards (SR), and a Department of Defense Breast Cancer Research Program pre-doctoral traineeship (#BC073318 to MJF). This study was funded, in part, by the National Institute of Health (#1R15CA125682-01 and #1R01CA140732-01A1 to SR), Illinois William E. McElroy Foundation (SR) and SIU-SOM Excellence in Medicine awards (SR), and by a Department of Defense Breast Cancer Research Program pre-doctoral traineeship (#BC073318 to MJF).

REFERENCES

1. Ahn KS, Aggarwal BB. Transcription factor NF-kappaB: a sensor for smoke and stress signals. *Ann N Y Acad Sci* 1056: 218–233, 2005.
2. Aizawa K, Suzuki T, Kada N, Ishihara A, Kawai-Kowase K *et al.* Regulation of platelet-derived growth factor-A chain by Kruppel-like factor 5: new pathway of cooperative activation with nuclear factor-kappaB. *J Biol Chem* 279: 70–76, 2004.
3. Alitalo K, Tammela T, Petrova TV. Lymphangiogenesis in development and human disease. *Nature* 438: 946–953, 2005.
4. Backhed F, Crawford PA, O'Donnell D, Gordon JI. Postnatal lymphatic partitioning from the blood vasculature in the small intestine requires fasting-induced adipose factor. *Proc Natl Acad Sci U S A* 104: 606–611, 2007.
5. Baluk P, Tammela T, Ator E, Lyubynska N, Achen MG *et al.* Pathogenesis of persistent lymphatic vessel hyperplasia in chronic airway inflammation. *J Clin Invest* 115: 247–257, 2005.
6. Baluk P, Yao LC, Feng J, Romano T, Jung SS *et al.* TNF-alpha drives remodeling of blood vessels and lymphatics in sustained airway inflammation in mice. *J Clin Invest* 119: 2954–2964, 2009.
7. Banerji S, Ni J, Wang SX, Clasper S, Su J *et al.* LYVE-1, a new homologue of the CD44 glycoprotein, is a lymph-specific receptor for hyaluronan. *J Cell Biol* 144: 789–801, 1999.
8. Beg AA, Sha WC, Bronson RT, Ghosh S, Baltimore D. Embryonic lethality and liver degeneration in mice lacking the RelA component of NF-kappa B. *Nature* 376: 167–170, 1995.
9. Beinke S, Ley SC. Functions of NF-kappaB1 and NF-kappaB2 in immune cell biology. *Biochem J* 382: 393–409, 2004.
10. Cariappa A, Liou HC, Horwitz BH, Pillai S. Nuclear factor kappa B is required for the development of marginal zone B lymphocytes. *J Exp Med* 192: 1175–1182, 2000.
11. Chang M, Lee AJ, Fitzpatrick L, Zhang M, Sun SC. NF-kappa B1 p105 regulates T cell homeostasis and prevents chronic inflammation. *J Immunol* 182: 3131–3138, 2009.
12. Charest-Marcotte A, Dufour CR, Wilson BJ, Tremblay AM, Eichner LJ *et al.* The homeobox protein Prox1 is a negative modulator of ERR{alpha}/PGC-1{alpha} bioenergetic functions. *Genes Dev* 24: 537–542, 2010.
13. Chen FE, Ghosh G. Regulation of DNA binding by Rel/NF-kappaB transcription factors: structural views. *Oncogene* 18: 6845–6852, 1999.
14. Chen Q, Dowhan DH, Liang D, Moore DD, Overbeek PA. CREB-binding protein/p300 co-activation of crystallin gene expression. *J Biol Chem* 277: 24081–24089, 2002.
15. Colotta F, Allavena P, Sica A, Garlanda C, Mantovani A. Cancer-related inflammation, the seventh hallmark of cancer: links to genetic instability. *Carcinogenesis* 30: 1073–1081, 2009.
16. Cui W, Tomarev SI, Piatigorsky J, Chepelinsky AB, Duncan MK. Mafs, Prox1, and Pax6 can regulate chicken betaB 1-crystallin gene expression. *J Biol Chem* 279: 11088–11095, 2004.
17. Dabdoub A, Puligilla C, Jones JM, Fritzsch B, Cheah KS *et al.* Sox2 signaling in prosensory domain specification and subsequent hair cell differentiation in the developing cochlea. *Proc Natl Acad Sci U S A* 105: 18396–18401, 2008.
18. Dyer MA, Livesey FJ, Cepko CL, Oliver G. Prox1 function controls progenitor cell proliferation and horizontal cell genesis in the mammalian retina. *Nat Genet* 34: 53–58, 2003.
19. Ferguson AR, Corley RB. Accumulation of marginal zone B cells and accelerated loss of follicular dendritic cells in NF-kappaB p50-deficient mice. *BMC Immunol* 6: 8, 2005.
20. Flister MJ, Wilber A, Hall KL, Iwata C, Miyazono K *et al.* Inflammation induces lymphangiogenesis through up-regulation of VEG-FR-3 mediated by NF-kappaB and Prox1. *Blood* 115: 418–429, 2010.
21. Furtado GC, Marinkovic T, Martin AP, Garin A, Hoch B *et al.* Lymphotoxin beta receptor signaling is required for inflammatory lymphangiogenesis in the thyroid. *Proc Natl Acad Sci U S A* 104: 5026–5031, 2007.
22. Ganta VC, Cromer W, Mills GL, Traylor J, Jennings M *et al.* Angiopoietin-2 in experimental colitis. *Inflamm Bowel Dis* 16: 1029–1039, 2010.
23. Gilmore TD. Introduction to NF-kappaB: players, pathways, perspectives. *Oncogene* 25: 6680–6684, 2006.
24. Gotaskie GE, Andreassi BF. Paclitaxel: a new antimitotic chemotherapeutic agent. *Cancer Pract* 2: 27–33, 1994.
25. Groger M, Loewe R, Holnthoner W, Embacher R, Pillinger M *et al.* IL-3 induces expression of lymphatic markers Prox-1 and podoplanin in human endothelial cells. *J Immunol* 173: 7161–7169, 2004.
26. Guo R, Zhou Q, Proulx ST, Wood R, Ji RC *et al.* Inhibition of lymphangiogenesis and lymphatic drainage via vascular endothelial growth factor receptor 3 blockade increases the severity of inflammation in a mouse model of chronic inflammatory arthritis. *Arthritis Rheum* 60: 2666–2676, 2009.
27. Henno A, Blacher S, Lambert C, Colige A, Seidel L *et al.* Altered expression of angiogenesis and lymphangiogenesis markers in the uninvolved skin of plaque-type psoriasis. *Br J Dermatol* 160: 581–590, 2009.
28. Hirakawa S, Brown LF, Kodama S, Paavonen K, Alitalo K, Detmar M. VEGF-C-induced lymphangiogenesis in sentinel lymph nodes promotes tumor metastasis to distant sites. *Blood* 109: 1010–1017, 2007.
29. Hoffmann A, Baltimore D. Circuitry of nuclear factor kappaB signaling. *Immunol Rev* 210: 171–186, 2006.
30. Hoffmann A, Natoli G, Ghosh G. Transcriptional regulation via the NF-kappaB signaling module. *Oncogene* 25: 6706–6716, 2006.
31. Hong YK, Foreman K, Shin JW, Hirakawa S, Curry CL *et al.* Lymphatic reprogramming of blood vascular endothelium by Kaposi sarcoma-associated herpesvirus. *Nat Genet* 36: 683–685, 2004.
32. Hong YK, Harvey N, Noh YH, Schacht V, Hirakawa S *et al.* Prox1 is a master control gene in the program specifying lymphatic endothelial cell fate. *Dev Dyn* 225: 351–357, 2002.

UNCORRECTED PROOF

33. Hou Y, Li F, Karin M, Ostrowski MC. Analysis of the IKKbeta/NF-kappaB signaling pathway during embryonic angiogenesis. *Dev Dyn* 237: 2926–2935, 2008.
34. Denis-Donini S, Dellarole A, Crociara P, Francese MT, Bortolotto V *et al.* Impaired adult neurogenesis associated with short-term memory defects in NF-kappaB p50-deficient mice. *J Neurosci* 28: 3911–3919, 2008.
35. Johnson LA, Jackson DG. Cell traffic and the lymphatic endothelium. *Ann N Y Acad Sci* 1131: 119–133, 2008.
36. Johnson NC, Dillard ME, Baluk P, McDonald DM, Harvey NL *et al.* Lymphatic endothelial cell identity is reversible and its maintenance requires Prox1 activity. *Genes Dev* 22: 3282–3291, 2008.
37. Kang S, Lee SP, Kim KE, Kim HZ, Memet S, Koh GY. Toll-like receptor 4 in lymphatic endothelial cells contributes to LPS-induced lymphangiogenesis by chemotactic recruitment of macrophages. *Blood* 113: 2605–2613, 2009.
38. Kataru RP, Jung K, Jang C, Yang H, Schwendener RA *et al.* Critical role of CD11b$^+$ macrophages and VEGF in inflammatory lymphangiogenesis, antigen clearance, and inflammation resolution. *Blood* 113: 5650–5659, 2009.
39. Kato A, Edwards MJ, Lentsch AB. Gene deletion of NF-kappa B p50 does not alter the hepatic inflammatory response to ischemia/reperfusion. *J Hepatol* 37: 48–55, 2002.
40. Kerjaschki D, Huttary N, Raab I, Regele H, Bojarski-Nagy K *et al.* Lymphatic endothelial progenitor cells contribute to de novo lymphangiogenesis in human renal transplants. *Nat Med* 12: 230–234, 2006.
41. Kunstfeld R, Hirakawa S, Hong YK, Schacht V, Lange-Asschenfeldt B *et al.* Induction of cutaneous delayed-type hypersensitivity reactions in VEGF-A transgenic mice results in chronic skin inflammation associated with persistent lymphatic hyperplasia. *Blood* 104: 1048–1057, 2004.
42. Kurland JF, Kodym R, Story MD, Spurgers KB, McDonnell TJ, Meyn RE. NF-kappaB1 (p50) homodimers contribute to transcription of the bcl-2 oncogene. *J Biol Chem* 276: 45380–45386, 2001.
43. Lavado A, Oliver G. Prox1 expression patterns in the developing and adult murine brain. *Dev Dyn* 236: 518–524, 2007.
44. Lo JC, Basak S, James ES, Quiambo RS, Kinsella MC *et al.* Coordination between NF-kappaB family members p50 and p52 is essential for mediating LTbetaR signals in the development and organization of secondary lymphoid tissues. *Blood* 107: 1048–1055, 2006.
45. Lu ZY, Yu SP, Wei JF, Wei L. Age-related neural degeneration in nuclear-factor kappaB p50 knockout mice. *Neuroscience* 139: 965–978, 2006.
46. Makinen T, Veikkola T, Mustjoki S, Karpanen T, Catimel B *et al.* Isolated lymphatic endothelial cells transduce growth, survival and migratory signals via the VEGF-C/D receptor VEGFR-3. *EMBO J* 20: 4762–4773, 2001.
47. Mancuso MR, Davis R, Norberg SM, O'Brien S, Sennino B *et al.* Rapid vascular regrowth in tumors after reversal of VEGF inhibition. *J Clin Invest* 116: 2610–2621, 2006.
48. Mishima K, Watabe T, Saito A, Yoshimatsu Y, Imaizumi N *et al.* Prox1 induces lymphatic endothelial differentiation via integrin alpha9 and other signaling cascades. *Mol Biol Cell* 18: 1421–1429, 2007.
49. Mounzer RH, Svendsen OS, Baluk P, Bergman CM, Padera TP *et al.* Lymphotoxin alpha contributes to lymphangiogenesis. *Blood*, ???: ???–???, 2010.
50. Mouta C, Heroult M. Inflammatory triggers of lymphangiogenesis. *Lymphat Res Biol* 1: 201–218, 2003.
51. Mouta CC, Nasser SM, di Tomaso E, Padera TP, Boucher Y *et al.* LYVE-1 is not restricted to the lymph vessels: expression in normal liver blood sinusoids and down-regulation in human liver cancer and cirrhosis. *Cancer Res* 61: 8079–8084, 2001.
52. Oliver G, Sosa-Pineda B, Geisendorf S, Spana EP, Doe CQ, Gruss P. Prox 1, a prospero-related homeobox gene expressed during mouse development. *Mech Dev* 44: 3–16, 1993.
53. Olszewski WL. The lymphatic system in body homeostasis: physiological conditions. *Lymphat Res Biol* 1: 11–21, 2003.
54. Ouaaz F, Arron J, Zheng Y, Choi Y, Beg AA. Dendritic cell development and survival require distinct NF-kappaB subunits. *Immunity* 16: 257–270, 2002.
55. Paavonen K, Puolakkainen P, Jussila L, Jahkola T, Alitalo K. Vascular endothelial growth factor receptor-3 in lymphangiogenesis in wound healing. *Am J Pathol* 156: 1499–1504, 2000.
56. Pan MR, Chang TM, Chang HC, Su JL, Wang HW, Hung WC. Sumoylation of Prox1 controls its ability to induce VEGFR3 expression and lymphatic phenotypes in endothelial cells. *J Cell Sci* 122: 3358–3364, 2009.
57. Panzer U, Steinmetz OM, Turner JE, Meyer-Schwesinger C, von RC *et al.* Resolution of renal inflammation: a new role for NF-kappaB1 (p50) in inflammatory kidney diseases. *Am J Physiol Renal Physiol* 297: F429–F439, 2009.
58. Paz-Priel I, Cai DH, Wang D, Kowalski J, Blackford A *et al.* CCAAT/enhancer binding protein alpha (C/EBPalpha) and C/EBPalpha myeloid oncoproteins induce bcl-2 via interaction of their basic regions with nuclear factor-kappaB p50. *Mol Cancer Res* 3: 585–596, 2005.
59. Petrova TV, Makinen T, Makela TP, Saarela J, Virtanen I *et al.* Lymphatic endothelial reprogramming of vascular endothelial cells by the Prox-1 homeobox transcription factor. *EMBO J* 21: 4593–4599, 2002.
60. Petrova TV, Nykanen A, Norrmen C, Ivanov KI, Andersson LC *et al.* Transcription factor PROX1 induces colon cancer progression by promoting the transition from benign to highly dysplastic phenotype. *Cancer Cell* 13: 407–419, 2008.
61. Ran S, Volk L, Hall K, Flister MJ. Lymphangiogenesis and lymphatic metastasis in breast cancer. *Pathophysiology* 17: 229–251, 2010.
62. Risebro CA, Searles RG, Melville AA, Ehler E, Jina N *et al.* Prox1 maintains muscle structure and growth in the developing heart. *Development* 136: 495–505, 2009.
63. Roberts N, Kloos B, Cassella M, Podgrabinska S, Persaud K *et al.* Inhibition of VEGFR-3 activation with the antagonistic antibody more potently suppresses lymph node and distant metastases than inactivation of VEGFR-2. *Cancer Res* 66: 2650–2657, 2006.
64. Saban MR, Memet S, Jackson DG, Ash J, Roig AA *et al.* Visualization of lymphatic vessels through NF-kappaB activity. *Blood* 104: 3228–3230, 2004.
65. Saccani S, Pantano S, Natoli G. Modulation of NF-kappaB activity by exchange of dimers. *Mol Cell* 11: 1563–1574, 2003.
66. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc* 3: 1101–1108, 2008.
67. Sha WC, Liou HC, Tuomanen EI, Baltimore D. Targeted disruption of the p50 subunit of NF-kappa B leads to multifocal defects in immune responses. *Cell* 80: 321–330, 1995.
68. Sledge GW Jr, Miller KD. Metastatic breast cancer: the role of chemotherapy. *Semin Oncol* 26: 6–10, 1999.
69. Sosa-Pineda B, Wigle JT, Oliver G. Hepatocyte migration during liver development requires Prox1. *Nat Genet* 25: 254–255, 2000.
70. Timmers L, van Keulen JK, Hoefer IE, Meijs MF, van MB *et al.* Targeted deletion of nuclear factor kappaB p50 enhances cardiac remodeling and dysfunction following myocardial infarction. *Circ Res* 104: 699–706, 2009.
71. Tomarev SI, Sundin O, Banerjee-Basu S, Duncan MK, Yang JM, Piatigorsky J. Chicken homeobox gene Prox 1 related to Drosophila prospero is expressed in the developing lens and retina. *Dev Dyn* 206: 354–367, 1996.

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72. Tong X, Yin L, Washington R, Rosenberg DW, Giardina C. The p50-p50 NF-kappaB complex as a stimulus-specific repressor of gene activation. *Mol Cell Biochem* 265: 171–183, 2004.
73. Vallabhapurapu S, Karin M. Regulation and function of NF-kappaB transcription factors in the immune system. *Annu Rev Immunol* 27: 693–733, 2009.
74. Veikkola T, Jussila L, Makinen T, Karpanen T, Jeltsch M *et al.* Signalling via vascular endothelial growth factor receptor-3 is sufficient for lymphangiogenesis in transgenic mice. *EMBO J* 20: 1223–1231, 2001.
75. Wang J, Kilic G, Aydin M, Burke Z, Oliver G, Sosa-Pineda B. Prox1 activity controls pancreas morphogenesis and participates in the production of ''secondary transition'' pancreatic endocrine cells. *Dev Biol* 286: 182–194, 2005.
76. Wigle JT, Harvey N, Detmar M, Lagutina I, Grosveld G *et al.* An essential role for Prox1 in the induction of the lymphatic endothelial cell phenotype. *EMBO J* 21: 1505–1513, 2002.
77. Wigle JT, Oliver G. Prox1 function is required for the development of the murine lymphatic system. *Cell* 98: 769–778, 1999.
78. Witmer AN, Dai J, Weich HA, Vrensen GF, Schlingemann RO. Expression of vascular endothelial growth factor receptors 1, 2, and 3 in quiescent endothelia. *J Histochem Cytochem* 50: 767–777, 2002.
79. Zhang J, Warren MA, Shoemaker SF, Ip MM. NFkappaB1/p50 is not required for tumor necrosis factor-stimulated growth of primary mammary epithelial cells: implications for NFkappaB2/p52 and RelB. *Endocrinology* 148: 268–278, 2007.
80. Zhang Q, Lu Y, Proulx ST, Guo R, Yao Z *et al.* Increased lymphangiogenesis in joints of mice with inflammatory arthritis. *Arthritis Res Ther* 9: R118, 2007.
81. Zumsteg A, Christofori G. Corrupt policemen: inflammatory cells promote tumor angiogenesis. *Curr Opin Oncol* 21: 60–70, 2009.